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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: S. Mori et al.

SERIAL NO: 09/674,337 EXAMINER: C. Fronda

FILED: July 26, 2001 GROUP: 1652

FOR: NICOTIANAMINE SYNTHASE AND GENE ENCODING THE SAME

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By Sharon Bizokas

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DECLARATION OF DR. SATOSHI MORI PURSUANT TO 37 CFR 1.132

I, Dr. Satoshi Mori, declare as follows:

1. I am a co-inventor of the above-referenced application, which I have assigned to the Japan Science and Technology Corporation. I am presently a Professor at the National Institution for Academic Degrees and University Evaluation, and I am also an Emeritus Professor of the University of Tokyo. I have been in both positions since 2003. Prior to my current positions, I was an Assistant Professor (1966-1990), an Associate Professor (1990-1994), and a Professor (1994-2003) of Applied Biological Chemistry at the University of Tokyo, Tokyo, Japan. I am an experienced Ph.D. with an extensive background in Plant Nutrition and Plant Molecular Physiology. Between 1987-2004, I authored or co-authored more than 120 scientific papers in my field. In

1999, I was awarded the Japan Agricultural Prize by the Agricultural Society of Japan and the Yomiuri Agricultural Prize by the Yomiuri Newspaper for "Development of the Transgenic Rice Tolerant to Iron Deficiency." In 1997, I was awarded the Prize of the Japanese Society of Soil Science and Plant Nutrition for "Studies on the Biosynthetic Pathway of Mugineic Acid." I hold B.S. (1964) and M.S. (1966) degrees in Agricultural Chemistry from the University of Tokyo, Tokyo, Japan. I hold a Ph.D. in Agricultural Chemistry (1981) from the University of Tokyo, Tokyo, Japan, where my dissertation was entitled, "Criticism of the Mineral Nutrition Theory."

2. The subject application discloses among other things and claims an isolated or purified enzyme exhibiting nicotianamine synthase activity, wherein the enzyme comprises the polypeptide having an amino acid sequence of SEQ ID NO: 1. It also discloses among other things and claims an isolated or purified enzyme exhibiting nicotianamine synthase activity, wherein the enzyme is a polypeptide having at least 50% identity with an amino acid sequence of SEQ ID NO: 1, comprising at least one of several consensus sequences of SEQ ID NO: 1 and having more than 25% of the nicotianamine synthase activity of an equivalent amount of the nicotianamine synthase activity of the enzyme of SEQ ID NO:1. Alternatively, the polypeptide has at least 90% identity with an amino acid sequence of SEQ ID NO: 1 or at least 95% identity with an amino acid sequence of SEQ ID NO: 1. It also discloses among other things and claims a mutated enzyme exhibiting nicotianamine synthase activity, wherein the enzyme is a polypeptide having more than 95% identity with an amino acid sequence of SEQ ID NO: 1, comprising at least one of several consensus sequences of SEQ ID NO: 1 and having more than 25% of the nicotianamine synthase activity of an equivalent amount of the nicotianamine synthase activity of the enzyme of SEQ ID NO:1.

3. Typically, iron is an essential nutrient of most plants, but may be absent or reduced in nutrient deficient soils, such as soils subject to erosion or over-farming, which poses a

problem in crop production worldwide, especially in calcareous soils, where iron is sparingly soluble due to the high soil pH. Iron is essential for the production of chlorophyll by the chloroplasts and for oxidative phosphorylation by the mitochondria. To acquire iron, graminaceous plants secrete iron (Fe) chelators, known as mugineic-acid (MA) family phytosiderophores, from their roots to absorb insoluble state iron (Fe(III)) from the soil. MAs dissolve iron in the rhizosphere, followed by reabsorption of the Fe(III)-MA complexes via the Strategy-II mechanism of iron acquisition. Nicotianamine (NA) is a key intermediate in the biosynthesis of MA, with nicotinanime synthase (NAS) catalyzing the trimerization of S-adenosinemethionine (SAM) into one molecule of NA (see "Background Art" on pp. 1-3 of the present application and Fig. 1). In graminaceous plants, tolerance to Fe deficiency is thought to depend on the amount of MAs secreted during Fe deficiency. For example, rice, which is a staple of many populous and/or developing countries, secretes small amounts of MAs and is susceptible to Fe deficiency, whereas barley secretes relatively high levels of MAs and can better tolerate Fe deficiency. Thus, it would be desirable to provide strains of plants having better tolerance of Fe deficiency in order to improve crop production and to support increasing populations.

4. The current invention addresses these concerns and many other issues as well, such as protection from contamination.

5. I have reviewed the United States Patent Office Action ("Office Action"), dated June 15, 2005, issued in connection with the subject application. As I understand the Office Action, the Patent Examiner has rejected certain claims of the application, alleging that the disclosure in the specification would not enable one of skill in the art to make or use the claimed nicotianamine synthases and that it would require undue experimentation for one of skill in the art to alter the claimed nicotianamine synthase to a nicotiananime synthase having more than 50% identity to the

sequence of SEQ ID NO: 1 and comprising 101 conserved amino acid residues, which the Patent Examiner states would require at least 164 amino acid residues to be changed (by deletion, insertion, substitution, or combinations thereof). As I understand the Office Action, the Patent Examiner has also rejected certain claims of the application, alleging that the disclosure in the specification would not enable one of skill in the art to make or use the claimed nicotianamine synthases and that it would require undue experimentation for one of skill in the art to make and screen for a nicotianamine synthase having more than 90% or 95% identity to the sequence of SEQ ID NO: 1, which the Patent Examiner states would require at least 33 amino acid residues to be changed (by deletion, insertion, substitution, or combinations thereof). As I understand the Office Action, the Patent Examiner also states that limiting the claims to recite specific amino acid sequences (SEQ ID Nos: 23-28) would not overcome the rejection alleging undue experimentation.

6. I disagree with these claim rejections.

7. The specification of the present application describes the process by which SEQ ID NO: 1 was obtained. Namely, nicotianamine synthase activity was compared between protein fractions obtained from the roots of barley plants grown under iron-deficient conditions with those from roots of control barley plants (pp. 7-8; Examples 1-3, pp. 19-22). Fractions containing nicotianamine synthase activity were identified, and partial amino acid sequences of candidate proteins in these fractions were obtained (pp. 7-9; Example 4, pp. 22-23; Fig. 4). These sequences were used to perform computer database searches which showed some similarity over short distances to rice and Arabidopsis genes of unknown function (p. 9). Primers were designed and used to obtain a series of clones: HvNAS1, HvNAS2, HvNAS3, HvNAS4, HvNAS5, HvNAS6, and HvNAS7 (pp. 9-11; Example 5, p. 23; Figs. 5-7). HvNAS1 has the nucleotide sequence SEQ ID NO: 2, the predicted amino acid sequence of which is SEQ ID NO: 1 (pp. 10-11). As shown in Table 1 (p. 11), the predicted amino acid sequences of the other six clones (HvNAS2-HvNAS7)

have between 61% and 74% identity to SEQ ID NO: 1 (Fig. 7). An inducible expression vector with the entire open reading frame (ORF) of HvNAS1 was created in a maltose binding protein expression vector and introduced into the XL1-Blue strain of Escherichia coli (E. coli) via transformation (pp. 11-13; Example 6, pp. 23-25). The protein was expressed and its activity assayed (pp. 11-13; Examples 2 and 6, pp. 19-20 and 23-25; Fig. 8) wherein the conversion of ^{14}C-labeled S-adenosyl methionine to nicotianamine was detected using thin layer chromatography. See Higuchi et al., Plant Soil, 178: 171-177 (1996) (copy provided).

8. In subsequent research, testing has revealed that the HvNAS1, HvNAS2, HvNAS3, HvNAS4, and HvNAS6 fusion proteins have nicotianamine synthase activity (see Higuchi et al., Plant Physiol. 119: 471-479 (1999); Higuchi et al., Soil Sci. Plant Nutr., 45: 681-691 (1999) (copies provided)). Except as noted, the results were largely qualitative. The HvNAS7 fusion protein has also been shown to exhibit nicotianamine synthase activity.

8. The specification of the present application describes the subsequent isolation and expression of nicotianamine synthase sequences from rice (Oryza sativa). We used restriction fragments of HvNAS1 to screen a cDNA library constructed from poly(A)+ RNA isolated from the roots of rice plants grown under iron-deficient conditions, and we obtained a clone (OsNAS1) having a full-length ORF (pp. 13-14). OsNAS1 has the nucleotide sequence SEQ ID NO: 16, the predicted amino acid sequence of which is SEQ ID NO: 15 (p. 14). The attached Reference Figure A shows a comparison between SEQ ID NO: 1 and other amino acid sequences, including SEQ ID NO: 15 (75% identity to SEQ ID NO: 1). An inducible expression vector with the entire open reading frame (ORF) of HvNAS1 was created and introduced into the XL1-Blue strain of Escherichia coli (E. coli) via transformation (p. 14; Example 6, pp. 23-25). The protein was expressed and its activity assayed in comparison with the activity from the protein expressed by the HvNAS1 expression vector (p. 14; Examples 2 and 6, pp. 19-20 and 23-25; Fig. 15). The results

demonstrate comparable relative activity between the expressed OsNAS1 protein and the expressed HvNAS1 protein (p. 14; Fig. 15). Thus, the polypeptide encoded by OsNAS1 has a more than 50% identity (i.e., 75%) to the polypeptide encoded by HvNAS1 and a relative nicotianamine synthase activity more than 25% (i.e., comparable to) that of the polypeptide of SEQ ID NO: 1.

9. In subsequent research, testing has revealed that the OsNAS2 and OsNAS3 fusion proteins also had nicotianamine synthase activity (see Higuchi et al., Plant J. 25: 159-167 (2001) and Inoue et al., Plant J. 36: 366-381 (2003) (copy provided)).

9. The specification of the present application describes the subsequent isolation and expression of nicotianamine sequences from Arabidopsis thaliana (Arabidopsis). By computer database searches, we had previously obtained Arabidopsis sequences similar to HvNAS1, and these sequences had been used to design primers (pp. 14-15). We performed PCR amplification of genomic DNA from Arabidopsis to obtain three nicotianamine synthase sequences (AtNAS1, AtNAS2, and AtNAS3) (pp. 14-15). We also created inducible expression vectors with each sequence and introduced them separately into the XL-1Blue strain of Escherichia coli (E. coli) via transformation (p. 15; Example 6, pp. 23-25). The proteins were expressed, and activity was detected (pp. 15-16).

10. In subsequent research, review using DNASIS (ver. 3.4; Hitachi Software Engineering Co. Ltd.) has revealed that the identity of AtNAS1, AtNAS2, and AtNAS3 in comparison with SEQ ID NO: 1 was 47.5%, 44.1%, and 48.5%, respectively. When expressed as fusion proteins in the above-described assay, all three show nicotianamine synthase activity. See Suzuki et al., Soil Sci. Plant Nutri. 45: 993-1002 (1999) (copy provided). A fourth sequence AtNAS4, having a sequence identity of 49.0% to SEQ ID NO: 1, also has been shown to have nicotianamine synthase activity.

10. In subsequent research, the present invention has been used to isolate additional nicotianamine synthases in rice (OsNAS2 and OsNAS3), corn (ZmNAS1, ZmNAS2, and ZmNAS3), and barley (NASHOR1 and NASHOR2). ZmNAS1 was cloned from a corn genomic library by using rice OsNAS1, while ZmNAS2 and ZmNAS3 were cloned from a corn cDNA library by using ZmNAS1 as a probe. The sequences are aligned with HvNAS1-HvNAS7 and OsNAS1, as shown in Reference Figure A, along with the respective percentage identity to SEQ ID NO: 1. The sequences shown in white with black background are conserved throughout the various nicotianamine synthases. In some cases where the sequences have not been conserved in all synthases, at least some of the differences are conservative. Examples of conservative differences include, but are not limited to, glutamate vs. aspartate, glutamine vs. asparagine, glutamate vs. glutamine, aspartate vs. asparagine, alanine vs. serine, alanine vs. threonine, and valine vs. isoleucine. The additional rice sequences, OsNAS2 and OsNAS3, are 75% and 62% identical to SEQ ID NO: 1, respectively, and these proteins, expressed as described above, have nicotianamine synthase activity. The corn sequences, ZmNAS1, ZmNAS2, and ZmNAS3, are 71%, 72%, and 66% identical to SEQ ID NO: 1. ZmNAS2 has additional sequence (insertion) not found in the other enzyme sequences shown. ZmNAS1 and ZmNAS3 have nicotianamine synthase activity, but ZmNAS2 does not. See Mizuno et al., Plant Physiol. 132: 1989-1997 (2003) (copy provided). The two additional barley sequences, NASHOR1 and NASHOR2, are 65% and 53% identical to SEQ ID NO: 1. Each of these sequences also contains at least one of the consensus sequences of SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, or SEQ ID NO: 28. With respect to the assays, except as noted in the application, the results were largely qualitative, but a visual comparison of the spots on the thin layer chromatograms with the spot derived from the HvNAS1 fusion clone (used as a positive control) showed that OsNAS2, OsNAS3, ZmNAS3 and AtNAS1 appear to have less activity than HvNAS1, while the other NAS fusion proteins (aside from HvNAS5 and ZmNAS2) had approximately the same activity as HvNAS1.

11. Therefore, both as shown in the application and subsequent to the filing of the application, the present invention has been used to isolate additional sequences of more than 50%, more than 90%, and more than 95% identity to SEQ ID NO: 1, having at least one consensus sequence of SEQ ID NO: 23 – SEQ ID NO: 28 and having at least 25% nicotianamine synthase activity relative to HvNAS1, without undue experimentation.

12. With respect to the Patent Examiner's rejection of claims to mutated enzymes, methods of mutagenesis were well-known when the application was filed. Methods of more systematic mutagenesis were also well-known, including site mutations, insertions, deletions, and combinations thereof using chemical or other mutagenesis with filamentous phage vectors (e.g., M13, pEMBL, lambda ZAP [λZAP]) or using a single-primer method or other oligonucleotide-mediated mutagenesis (single or multiple point mutations, insertions, deletions), a gapped duplex method, a couple priming and cyclic selection method, the Kunkel method, a strand selection method, transformation with oligonucleotides, looping in or looping out methods, nested deletions, and other methods (Sambrook et al., Molecular Cloning: A Laboratory Manual (2nd ed.), ch. 15, Cold Spring Harbor Laboratory Press (Cold Spring Harbor, NY: 1989); Old & Primrose, Principles of Gene Manipulation: An Introduction to Genetic Engineering (4th ed.), pp. 75-98 (Blackwell Scientific Publications (Boston: 1989)). Examples of random mutagenesis, including mutagenesis in response to stressful conditions (e.g., elevated temperatures), are reviewed by Arnold (Design by directed evolution. Acc. Chem. Res. 31: 125-131 (1998)), a copy of which is attached. Random mutagenesis can be achieved, for example, by forced misincorporation during DNA synthesis, by chemical mutagenesis, or by exposure to radiation. Forced misincorporation is frequently performed by a modified, error-prone polymerase chain reaction (PCR), such as by using a Taq DNA polhymerase that is error prone and lacking in proofreading capability. The error rate can be increased further by modifying the reaction buffer (e.g., Mn^{+2} concentration) or by using

unbalanced dNTP concentrations. These techniques were known in the art when the application was filed.

13. As an another example, we recently used error-prone PCR for random mutagenesis and screening techniques to obtain a series of mutants in work on a different plant enzyme (Oki et al., "Directed evolution of yeast ferric reductase to produce plants with tolerance to iron deficiency in alkaline soils," Soil Sci. Plant Nutr. 50 (7): 1159-1165 (2004)). A copy of this reference is provided. Although this reference was published after the filing of the application, methods of error-prone PCR and screening were known prior to the filing of the application.

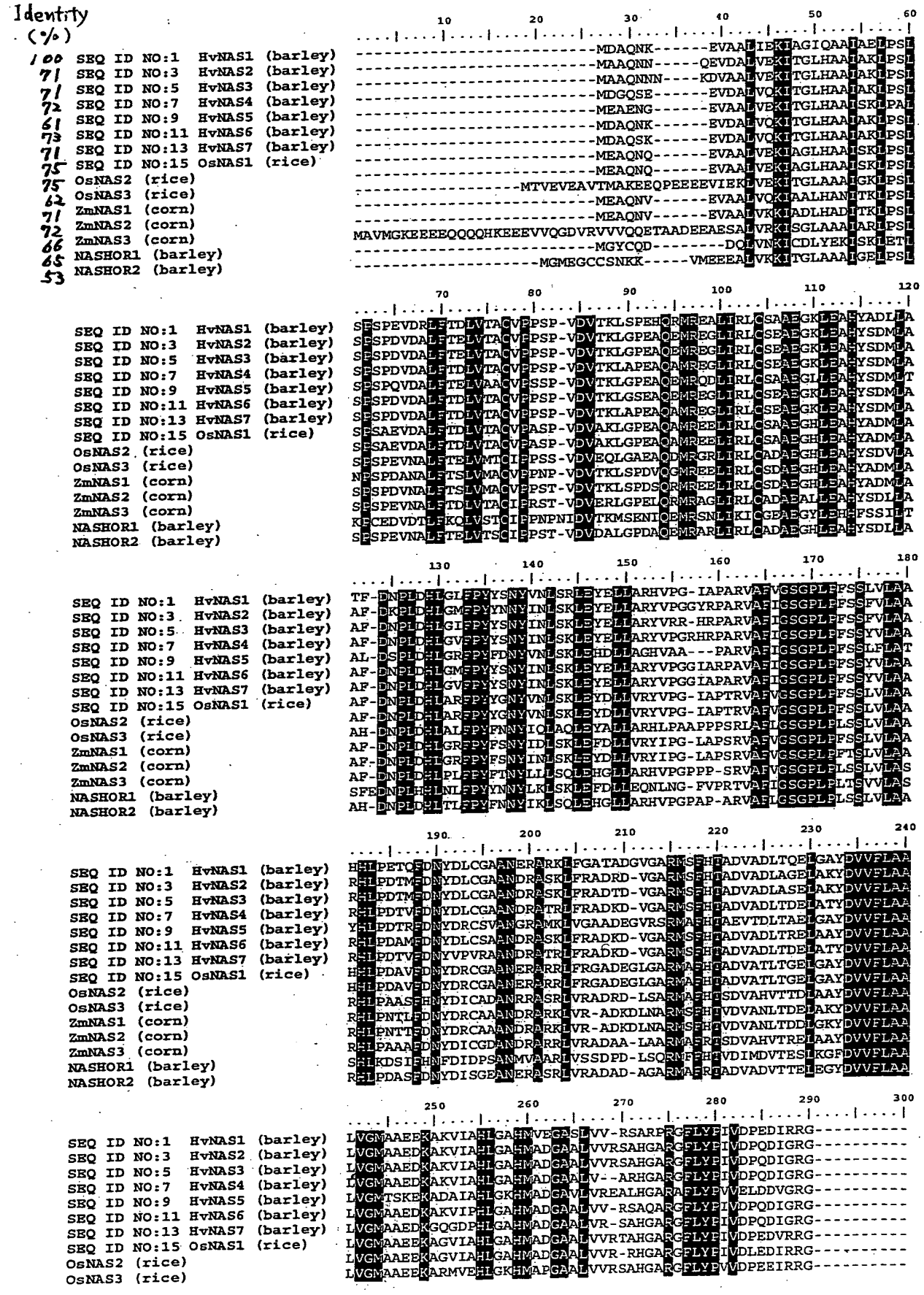
14. With the knowledge of the present invention, one of ordinary skill in the art could manipulate a nucleotide sequence, such as by using primer-direceted mutagenesis or one of the other techniques outlined above, to produce a nicotianamine synthase having at least 25% relative activity compared to HvNAS1, but without having a sequence identical to SEQ ID NO: 1.

15. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, and that such willful false statements may jeopardize the validity of the application on any patent issued thereon.

Date: Nov. 20, 2005

Satoshi Mori

Satoshi Mori, Ph.D.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61 SEQ ID NO:9 HvNAS5 (barley)
73 SEQ ID NO:11 HvNAS6 (barley)
71 SEQ ID NO:13 HvNAS7 (barley)
75 SEQ ID NO:15 OsNAS1 (rice)
75 OsNAS2 (rice)
62 OsNAS3 (rice)
71 ZmNAS1 (corn)
72 ZmNAS2 (corn)
66 ZmNAS3 (corn)
65 NASHOR1 (barley)
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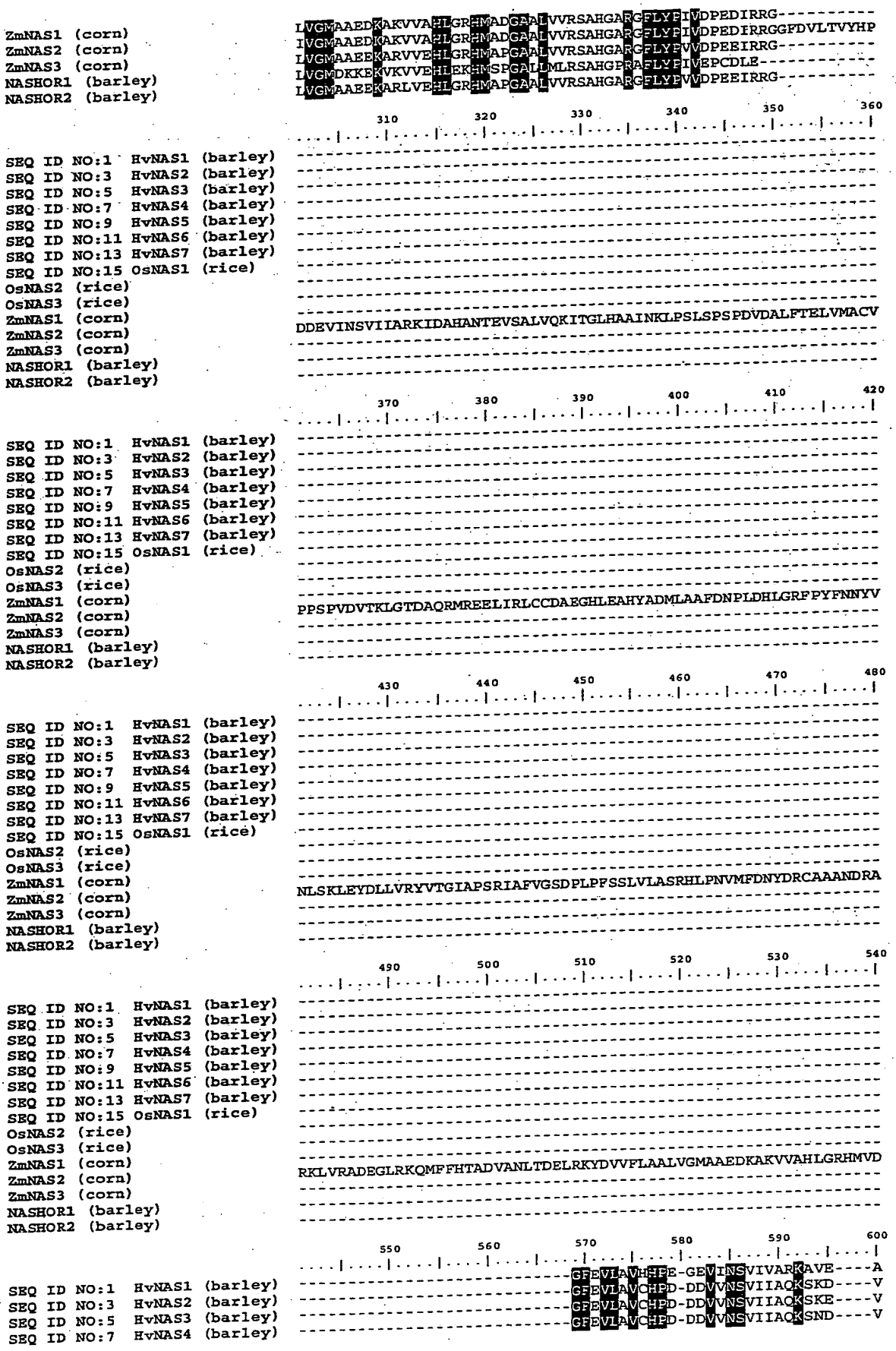
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ZmNAS3 (corn)
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SEQ ID NO:3 HvNAS2 (barley)
SEQ ID NO:5 HvNAS3 (barley)
SEQ ID NO:7 HvNAS4 (barley)
SEQ ID NO:9 HvNAS5 (barley)
SEQ ID NO:11 HvNAS6 (barley)
SEQ ID NO:13 HvNAS7 (barley)
SEQ ID NO:15 OsNAS1 (rice)
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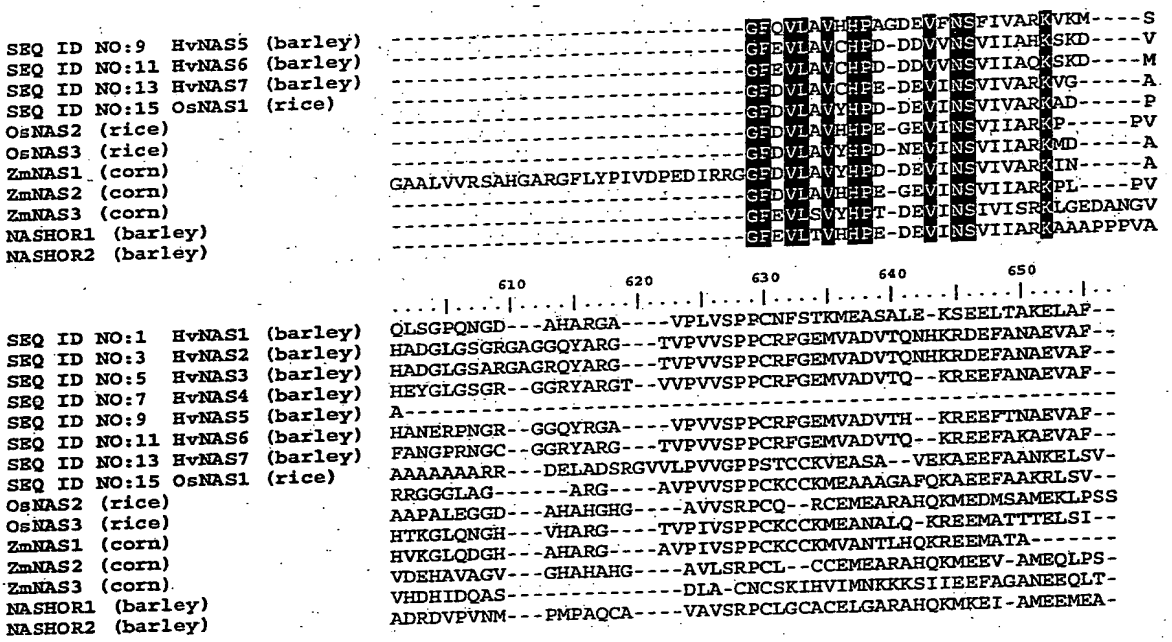
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ZmNAS3 (corn)
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NASHOR2 (barley)
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630
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650
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SEQ ID NO:11 HvNAS6 (barley)
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SEQ ID NO:15 OsNAS1 (rice)
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OsNAS3 (rice)
ZmNAS1 (corn)
ZmNAS2 (corn)
ZmNAS3 (corn)
NASHOR1 (barley)
NASHOR2 (barley)

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The role of nicotianamine synthase in response to Fe nutrition status in Gramineae

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Abstract

Nicotianamine is an intermediate for the biosynthesis of mugineic acid-family phytosiderophores (MAs) in the Gramineae and a key substance for iron metabolism in dicots. Nicotianamine synthase catalyzes the formation of nicotianamine from S-adenosylmethionine. Nicotianamine synthase activity was induced in barley roots at the 3rd day after withholding Fe supply and declined within one day followmg the supply of Fe^{3+}-epihydroxymugineic acid. The induction of nicotianamine synthase activity by Fe-deficiency was observed also in sorghum, maize, and rye, and the level of nicotianamine synthase activity was highly associated with the MAs secreted among graminaceous plant tested. Therefore, the nicotianamine synthase gene may be a suitable candidate for making a transgenic plant tolerant to Fe-deficiency.

Abbreviations: p-APMSF–(*p*-amidinophenyl) methanesulfonylfluoride hydrochloride, NA–nicotianamine, DMA–2′-deoxymugineic acid, E-64–*trans*-epoxysuccinyl-leucylamido-(4-guanidino) butane, epiHMA–3-epihydroxymugineic acid, MAs–mugineic acid-family phytosiderophores which include deoxymugineic acid, mugineic acid, hydroxymugineic acid, epihydroxymugineic acid and avenic acid, PVP–polyvinylpyrrolidone, SAM–S-adenosylmethionine.

Introduction

Grasses (graminaceous plants) excrete natural iron-chelators, called phytosiderophores, from the roots to solubilize the sparingly soluble iron in the rhizosphere, and the amount of the secreted phytosiderophores increases under Fe-deficiency stress. This iron acquisition mechanism of graminaceous plants is called 'Strategy II ' compared with 'Strategy I' of non-graminaceous plants (Römheld, 1987). The mugineic acid family (MAs) is the only example of phytosiderophores known so far (Takagi, 1976).

The biosynthetic pathway of MAs involves the conversion of three S-adenosylmethionine (SAM) molecules to form one nicotianamine molecule, which is then converted to deoxymugineic acid (DMA) by transamination and reduction at the 3″-carbon (Mori and Nishizawa, 1987; Shojima et al., 1989a, 1990). A subsequent series of hydroxylations of DMA may lead to other members of MAs (Fig.1) (Ma and Nomoto, 1993; Mori et al., 1990; Mori and Nishizawa, 1989).

Nicotianamine (NA) is a key substance in the pathway of the biosynthesis of MAs. Although NA is widely distributed among the plants of both 'strategy I' and 'strategy II' (Fushiya et al., 1982; Scholz et al., 1992; Shojima et al., 1989b), MAs have been found only in graminaceous plants until now. Therefore, the biosynthetic steps from NA to DMA and to the other MAs can be thought to be evolutionally acquired by the 'strategy II' plants, Gramineae.

We have previously reported partial purification and some characteristics of nicotianamine synthase (Higuchi et al., 1994). Nicotianamine synthase activity was detected mainly in roots, and induced by

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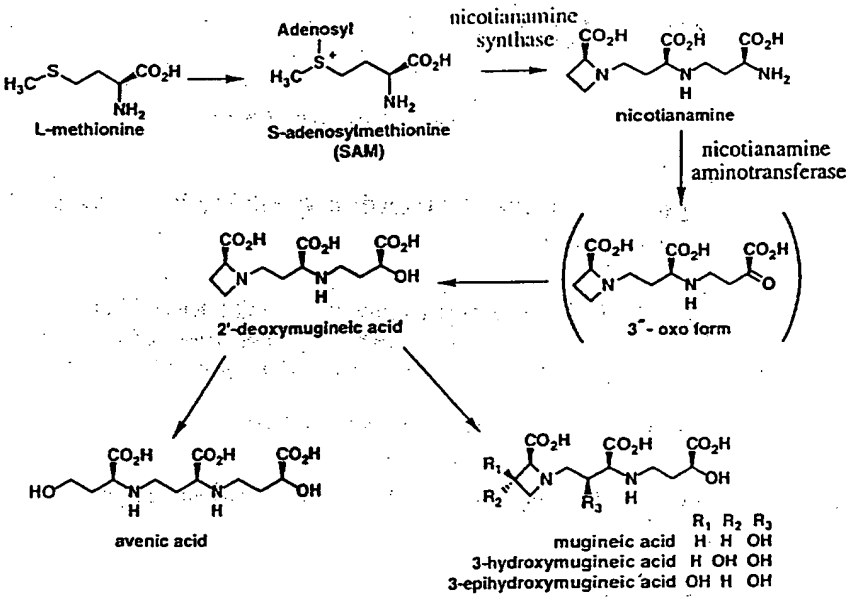

Fig. 1. Biosynthetic pathway of mugineic acids.

Fe-deficiency in barley (Higuchi et al., 1994). Here we describe the distribution in graminaceous plants, detailed induction and suppression profiles of nicotianamine synthase, and research on potent protease inhibitors to this enzymes. The role of nicotianamine synthase in Fe nutrition of graminaceous plant is also discussed.

Materials and methods

Plant material

Barley seeds (*Hordeum vulgare* L. cv Ehimehadaka-mugi no.1) were germinated on wet filter paper and transferred into a standard culture solution in a glass house at natural temperature under natural light. The composition of the standard solution was 7×10^{-4} *M* K_2SO_4, 1×10^{-4} *M* KCl, 1×10^{-4} *M* KH_2PO_4, 2×10^{-3} *M* $Ca(NO_3)_2$, 5×10^{-4} *M* $MgSO_4$, 1×10^{-5} *M* H_3BO_3, 5×10^{-7} *M* $MnSO_4$, 5×10^{-7} *M* $ZnSO_4$, 2×10^{-8} *M* $CuSO_4$, 1×10^{-8} *M* $(NH_4)_6Mo_7O_{24}$, and 1.5×10^{-4} *M* Fe-EDTA. The pH of the culture solution was adjusted to 5.5 by 0.5 *M* HCl every day. Plants (3 plants×14 holes) were cultured in a 20-L plastic container. When the third leaf emerged, the plants were transferred to the culture solution which contained all other nutrients except Fe. The culture solution was always prepared in deionized water. Iron-sufficient plants were cultured simultaneously with Fe-deficient plants in the standard culture solution with adequate Fe. The culture solutions were renewed every week. Two weeks after transplanting, when severe iron chlorosis appeared on the 4th and 5th leaves, the roots were harvested, crushed under liquid N_2 and stored at – 80 °C until use.

Chemicals

S-Adenosyl-L-methionine,-[carboxyl-^{14}C) (1.8 GBq mmol^{-1}) was purchased from Du Pont Company. [^{14}C]NA was synthesized from [^{14}C]SAM following the method of Shojima et al. (1989a). Antipain, E-64, leupeptin, chymostatin, pepstatin and trypsin inhibitor were purchased from Sigma Chemical Co.

Enzyme assay system

The cell-free system reported previously by Shojima et al. (1989a) was modified (Higuchi et al., 1994). Enzyme in the reaction buffer (50 m*M* Tris, 1 m*M* EDTA, 3 m*M* DTT, pH 8.7 with HCl) was concentrated by gel filtration or ultrafiltration using an Ultra-

free C3LGC NMWL10000 or Ultrafree-PC (LGC) NMWL10000 (Millipore Co.) or an NAP Column (Pharmacia LKB). Antipain was added to the enzyme solution at 20 $\mu g\ mL^{-1}$, and [^{14}C]SAM was added to the enzyme solution at the final concentration of 20 μM. After 15 min incubation at 25 °C, the enzyme reaction was stopped by adding ethanol to a final concentration of 50%(v/v). [^{14}C]NA was separated by TLC. Ten μL of the reaction mixtures and [^{14}C]NA were spotted on a silica gel TLC plate LK6 (Whatman), and the plates were developed with phenol:n-butanol:formate:water (12:3:2:3 v/v). The radioactivity of [^{14}C]NA was detected by an Image Analyzer BAS2000 (Fuji Film). The protein content was estimated using a Protein Assay Kit with Standard I (Bio Rad).

Extraction of nicotianamine synthase

Subsequent operations were performed at 0 to 4 °C. Fresh whole root tissues (0.5 g) were homogenized with a mortar and a pestle in 1 mL of 0.2 *M* Tris/HCl buffer (pH 8.0) containing 10 m*M* EDTA, 5% (w/v) PVP insoluble, 5% (v/v) glycerol, 0.1 m*M* p-APMSF, 100 $\mu g\ mL^{-1}$ antipain (or 1 m*M* E-64), and 10 m*M* DTT. The homogenate was centrifuged at 8000×*g* for 20 min.

Nicotianamine synthase activity in cereals

Barley (*Hordeum vulgare* L. cv Ehimehadakamugi No. 1), rice (*Oryza sativa* L. cv Koshihikari), maize (*Zea mays* L. cv Alice), sorghum (*Sorghum bicolor* (L.) MOENCH. cv Big jim), and wheat (*Triticum aestivum* L. cv Chinese Spring) were germinated on wet filter paper and transferred to the standard culture solution in a growth chamber with 19 °C/14 h-light and 14 °C/10 h - dark periods for barley and wheat, or with 27 °C/14 h - light and 22 °C/10 h - dark periods for maize, sorghum and rice (Kanazawa et al., 1994). When the third leaf (barley, wheat and rice), the fifth leaf (maize) or the sixth leaf (sorghum) emerged, the plants were transferred to the Fe-free culture solution. Control plants with adequate Fe were simultaneously transferred to fresh standard culture solution. Seven days after transplanting, the root washings were collected. The root washings were collected for 3 h after lighting-up using deionized water, because MAs are known to be excreted after sunrise under natural conditions (Takagi et al., 1984). MAs in the root washings were analyzed by HPLC according to the method of Mori et al. (1987).

Table 1. The effects of protease inhibitors in extraction buffer on nicotianamine synthase activity. All extraction buffer contained 10 m*M* EDTA and 0.1 m*M* p-APMSF. A 0.5 g fresh weight sample of Fe-deficient barley roots was extracted with 1 mL of each extraction buffer and applied to a Butyl TOYOPEARL column. The maximum nicotianamine synthase activity is indicated as 100%

Protease inhibitor	Nicotianamine synthase activity
Antipain 100 $\mu g\ mL^{-1}$	100%
E-64 1 m*M*	100%
Leupeptin 100 $\mu g\ mL^{-1}$	22%
Chymostatin 100 $\mu g\ mL^{-1}$	38%
Pepstatin 100 $\mu g\ mL^{-1}$	27%
Trypsin inhibitor (soybean) 100 $\mu g\ mL^{-1}$	20%
None	21%

Next day, roots were harvested, dipped in liquid nitrogen, crushed and stored at −80 °C until use. Aliquots of 0.5 g of the frozen roots were homogenized with 100 $\mu g\ m^{-1}$ antipain. The supernatant was applied to a hydrophobic resin, TSK gel Butyl-TOYOPEARL 650M (Fractogel TSK Butyl-650M, Merck) 1 mL column equilibrated with 20 m*M* Tris/HCl buffer (pH 8.0) containing 1 m*M* EDTA, 0.1 m*M* p-APMSF, 3 m*M* DTT and 0.4 *M* $(NH_4)_2SO_4$. Nicotianamine synthase activity was eluted with 3 mL of 1% glycerol containing 0.1 m*M* p-APMSF and 3 m*M* DTT. The enzyme activity was determined in duplicate.

Time course study of enzyme induction and suppression

Roots of 9 barley plants per culture were harvested at 0, 1st, 3rd, 5th and 7th day of the Fe starvation. Before harvesting, the root-washings were collected for 1 h starting 1 h after sunrise (Takagi et al., 1994). MAs in root-washings were determined as described previously (Mori and Nishizawa, 1987). The whole roots of 9 plants were homogenized in liquid N_2 and each 0.5 g fresh weight sample was used for the determination of the enzyme activity. Barley plants were grown under Fe-deficient conditions for 10 days then 1 mg kg^{-1} Fe was supplied as epiHMA-Fe^{3+} or EDTA-Fe^{3+}. After the addition of Fe, roots of 3 plants per culture were harvested at 0, 6, 12, 24 and 48 h. The whole roots of 3 plants were homogenized in liquid N_2, and each 0.5 g fresh weight root sample was homogenized with

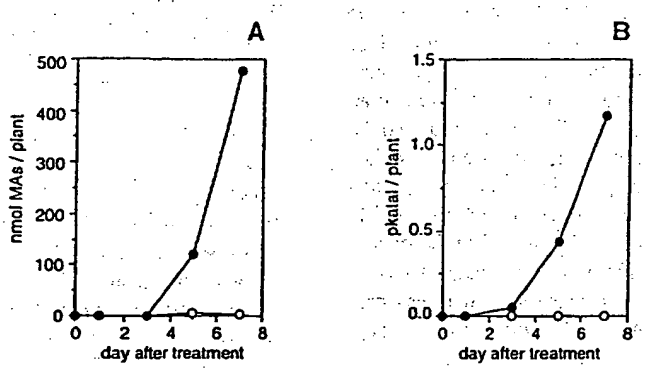

Fig. 2. Time course of induction of MAs secretion and NA synthase in barley. Each value is mean (n=2). A: MAs secretion, B: NA synthase activity. The root washings and roots materials for enzyme assay were obtained from each of 9 barley plants. closed circle : Fe-deficient barley open circle: Fe-sufficient barley.

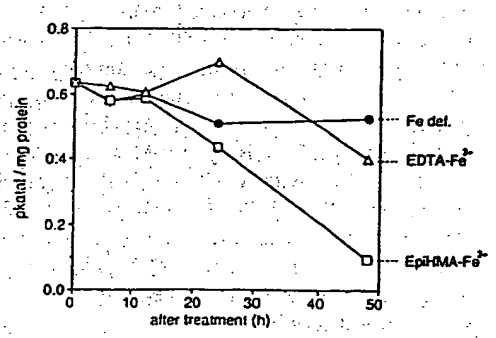

Fig. 3. Time course of suppression of NA synthase in barley. Each value is mean (n=2). closed circle: Fe-deficiency, open triangle: EDTA-Fe^{3+}, open square: epiHMA-Fe^{3+}.

1 m*M* E-64. The enzyme activity was determined in duplicate.

Results

Screening of effective protease inhibitor for protection of nicotianamine synthase activity

In a previous paper, nicotianamine synthase activity was determined using root tips (Higuchi et al.,1994; Shojima et al., 1990). Since the weight of root tips was small, it was difficult to estimate the enzyme activity per plant using only root tips. In the present investigation, we intended to clarify the time course of induction and repression of NA activity and the relationship between the enzyme activity and mugineic acids secretion. For that purpose it was necessary to determine the enzyme activities in whole roots. However, in the previous studies the enzyme activity was lost when we used whole roots. It was supposed that nicotianamine synthase was very sensitive to some endogenous protease activity, and that compared to root tips, the old root tissues contained elevated protease activities. Thus, to establish an enzyme assay system from whole root tissues, several protease inhibitors were tested. Antipain and E-64 were found to be the most effective (Table 1). Chymostatin was also effective but less than antipain and E-64. This suggested that thiol protease(s) such as papain might digest nicotianamine synthase in crude extracts.

Time course of enzyme induction and suppression in barley roots

Previously we reported that nicotianamine synthase was induced within a week after Fe-deficiency treatment and suppressed within one week after addition of Fe (Higuchi et al., 1994). In this work, we determined a more detailed profile of enzyme induction and suppression. The increase in enzyme activity was associated with the increase in secretion of MAs (Fig. 2). The enzyme induction appeared clearly 3 day after Fe-deficiency treatment.

After epiHMA-Fe^{3+} treatment, nicotianamine synthase activity was constant for 12 h, then suppressed at 24 h, and decreased to the level of an Fe-sufficient plant after 48 h (Fig. 3). In case of EDTA-Fe^{3+}, the suppression was delayed by one day in comparison with epiHMA-Fe^{3+}. This is in agreement with previous results that the rate of mugineic acid-Fe^{3+} uptake was

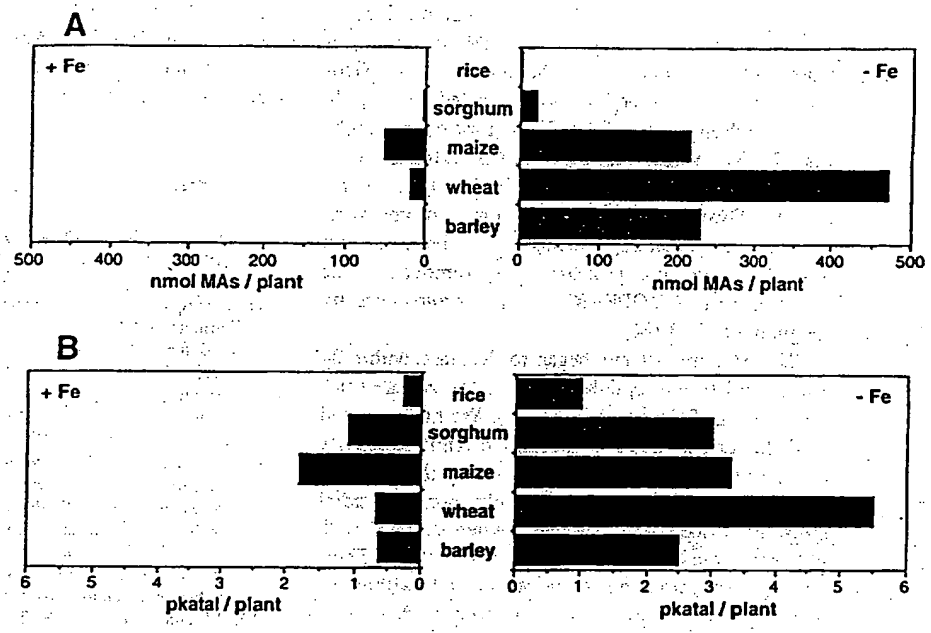

Fig. 4. Induction of NA synthase activity and mugineic acids secretion in several cereals. A: MAs secretion B: NA synthase activity. The root washings and root materials for enzyme assay came from 42 barley plants, 42 oat plants, 42 wheat plants, 7 maize plants, 14 barley plants, 42 oat plants, 42 wheat plants, 7 maize plants, 14 sorghum plants and 45 rice plants. Each value is mean (n=2). The enzyme assay data were reproducible, and this figure shows a typical experiment.

very much higher than that of EDTA-Fe^{3+} (Marschner et al., 1986; Takagi, 1984). A recent report suggests that Fe of EDTA-Fe^{3+} requires ligand-exchange with DMA, before absorption by maize roots (Yehuda et al., 1995).

Nicotianamine synthase activity in cereals

Nicotianamine synthase activity was investigated in barley, wheat, maize, sorghum, and rice. The detailed experimental conditions (shoot chlorosis and age) of those plant materials have been given in our previous work (Kanazawa et al., 1994). Nicotianamine synthase activity was significantly induced by Fe-deficiency in all species, and correlated to the amount of secreted MAs. A representative example is shown in Figure 4. In our earlier study we obtained similar results using the same five gramineous species.

Discussion

Tolerance to Fe-deficiency in grasses depends upon the secreted amount of phytosiderophores (Marschner et al., 1986; Singh et al., 1993). We demonstrated close relationships between nicotianamine synthase activity and the secretion of MAs. On the other hand, a similar relationship to MAs secretion has also been reported in case of nicotianamine aminotransferase (Kanazawa et al., 1994). However, nicotianamine synthase is, of course, upstream of nicotianamie aminotransferase in the MAs biosynthetic pathway. Increase of nicotianamine synthase activity will thus have a greater impact on MAs synthesis than those of other downstream enzymes.

Nicotianamine synthase activity was significantly induced by Fe-deficiency in 5 graminaceous plants (Fig. 4). However, in contrast to induction in graminaceous plants, nicotianamine synthase activity was not induced by Fe-deficiency in tobacco (Higuchi et al., 1995), tomato and soybean (unpublished data). In future, comparison of the nucleotide sequence of the genomic DNA of nicotianamine synthase between

graminaceous plants and dicot plants may lead to better understanding of the regulatory mechanism of nicotianamine synthesis.

Antipain and E-64 were potent protectors of nicotianamine synthase. Antipain inhibited thiol protease reversibly and E-64 inhibited irreversibly. Nicotianamine synthase activity was detected from crude extracts when extracted with E-64 (time course study, Fig. 2, 3). When the enzyme was extracted with the addition of antipain, the activities were detected after Butyl TOYOPEARL column chromatography (several cereal experiments, Fig. 4) because most part of endogenous proteases were removed at the step of Butyl TOYOPEARL column chromatography (Higuchi et al., 1994).

The enzyme activity began to decrease within 24 h after addition of epiHMA-Fe^{3+} to the culture solution of Fe-deficient barley (Fig. 3). We had expected much faster decline of nicotianamine synthase activity in response to the Fe re-supply. Usually the transport of Fe from root epidermis to vascular bundle ("radial transport") takes less than one h when supplied with mugineic acid-Fe^{3+} (Mihashi and Mori, 1989). Thus, Fe will enter into cortex or epidermal cells within one h, and the repression of nicotianamine synthase gene expression should occur in less than 24 h. In fact, iron induced transcription of ferritin gene in cultured tobacco cells occurs within 6 h after overloading of Fe in the culture medium (Lescure et al., 1991). Why did it take 24 h ? If roots receive the information or signal about Fe nutrition status from leaves, instead of roots, this slow decline of nicotianamine synthase in response to Fe re-supply may be reasonable. For example, Walter et al. (1994) suggested that Fe-deficiency in shoot, caused by Zn-deficiency, induced MAs secretion from barley roots even under Fe-sufficient condition.

We demonstrated that nicotianamine synthase activity was strictly regulated by Fe nutrition status and associated with MAs secretion. Thus, the nicotianamine synthase gene may be one of the candidates for resistant gene to Fe-deficiency. We have previously purified nicotianamine synthase to yield one band on SDS-PAGE gel by using leupeptin as a protease inhibitor (Higuchi et al., 1994). However, more suitable protease inhibitors, like antipain or E-64, were found in this experiment. This will contribute to the complete purification of nicotianamine synthase and will open the way in future to clone this gene after sequencing its peptides.

Acknowledgements

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Cloning of Nicotianamine Synthase Genes, Novel Genes Involved in the Biosynthesis of Phytosiderophores

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Nicotianamine synthase (NAS), the key enzyme in the biosynthetic pathway for the mugineic acid family of phytosiderophores, catalyzes the trimerization of *S*-adenosylmethionine to form one molecule of nicotianamine. We purified NAS protein and isolated the genes *nas1*, *nas2*, *nas3*, *nas4*, *nas5-1*, *nas5-2*, and *nas6*, which encode NAS and NAS-like proteins from Fe-deficient barley (*Hordeum vulgare* L. cv Ehimehadaka no. 1) roots. *Escherichia coli* expressing *nas1* showed NAS activity, confirming that this gene encodes a functional NAS. Expression of *nas* genes as determined by northern-blot analysis was induced by Fe deficiency and was root specific. The NAS genes form a multigene family in the barley and rice genomes.

Graminaceous plants that adopt the Strategy II mechanism of Fe acquisition (Römheld, 1987) secrete Fe chelators, called phytosiderophores, from their roots to solubilize sparingly soluble Fe in the rhizosphere. The amount of phytosiderophore secreted increases dramatically under Fe-deficiency stress. To our knowledge, the MA is the only class of phytosiderophore so far identified in plants (Takagi, 1976). Tolerance to Fe deficiency in graminaceous plants is thought to depend on the quantity of MAs secreted by plants under Fe-deficiency stress (Takagi et al., 1984; Römheld and Marschner, 1986; Marschner et al., 1987; Mori et al., 1987, 1988; Kawai et al., 1988; Mihashi and Mori, 1989; Singh et al., 1993). Of the graminaceous plants, rice secretes the least MAs and is the species most susceptible to Fe deficiency in calcareous soils. Transgenic rice overexpressing the genes of MA biosynthesis under Fe deficiency should secrete greater amounts of MAs and tolerate Fe deficiency.

The biosynthetic pathway for MAs has been determined (Fig. 1). SAM is synthesized from Met by SAM synthetase. Subsequently, three molecules of SAM are combined to form one molecule of NA by NAS. NA is then converted to [3''-keto acid] by NAAT, and deoxymugineic acid is synthesized by the subsequent action of a reductase. A further series of hydroxylation steps produces the other MA members from deoxymugineic acid (Fig. 1; Mori and Nishizawa, 1987; Shojima et al., 1989, 1990; Ma and Nomoto, 1993). Three cDNAs encoding SAM synthetase from barley (*Hordeum vulgare* L.) roots have been cloned (Takizawa et al., 1996; accession nos. D63835, D85273, and D85238), but these genes are not induced by Fe deficiency. Recently, NAAT was purified and two NAAT cDNAs (*naat-A* and *naat-B*) were cloned from Fe-deficient barley roots (Takahashi et al., 1997). *naat-A* expression was shown to be specifically induced in Fe-deficient roots. A clone encoding the putative mugineic acid synthase *Ids3*, which converts deoxymugineic acid to mugineic acid and is strongly induced by Fe deficiency, was cloned from the barley genome using differential hybridization (Nakanishi et al., 1993; accession no. D37796).

The synthesis of NA from SAM is similar to polyamine synthesis from decarboxy-SAM. In contrast to spermidine synthase (Pajula et al., 1979), however, NAS catalyzes the polymerization of three SAM molecules with the release of adenine and the azetidine ring formation at the same time (Fig. 2). Therefore, NAS is a novel type of enzyme. Previously, we reported the partial purification of NAS from the roots of Fe-deficient barley and showed that NAS activity was induced under Fe-deficiency stress (Higuchi et al., 1994, 1995; Kanazawa et al., 1995). Since NAS is vulnerable to degradation by proteases using the purification method previously described, it has been difficult to purify sufficient quantities to determine its partial amino acid sequence. In this paper we describe an improved purification procedure for NAS that allowed us to obtain sufficient quantities for amino acid sequencing. Subsequently, we cloned and characterized several genes encoding NAS or NAS-like proteins.

MATERIALS AND METHODS

Preparation of Plant Material

Barley (*Hordeum vulgare* L. cv Ehimehadaka no. 1) was grown in hydroponic culture as previously described

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Abbreviations: Chaps, 3-[(3-cholamidopropyl)dimethyl-ammonio] propanesulfonic acid; CNBr, cyanogen bromide; E-64, trans-epoxysuccinyl-leucylamido-(4-guanidino)butane; MAs, mugineic acid family; NA, nicotianamine; NAAT, nicotianamine aminotransferase; NAS, nicotianamine synthase; p-APMSF, (*p*-amidinophenyl)methanesulfonyl fluoride; SAM, *S*-adenosylmethionine.

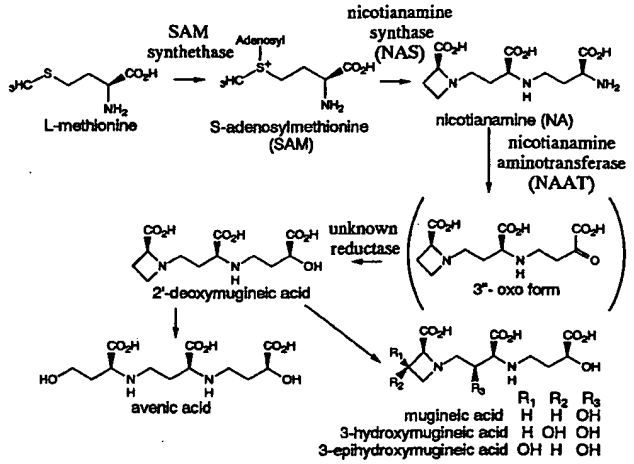

Figure 1. Biosynthetic pathway of the MAs of phytosiderophores.

(Higuchi et al., 1994). Two weeks after Fe-deficiency treatment, when severe Fe chlorosis appeared on the fourth or fifth leaf, the roots were harvested, crushed in liquid N_2, and stored at −80°C until use.

Assay of NAS Activity

Enzyme solutions were equilibrated with reaction buffer (50 mM Tris, 1 mM EDTA, 3 mM DTT, 10 μM p-APMSF, and 10 μM E-64, pH 8.7) and concentrated by ultrafiltration using Ultrafree C3LGC NMWL10000 (Millipore). The details of the method used for detecting NAS activity were described by Higuchi et al. (1996a).

Purification of NAS Proteins

The roots were crushed into a fine powder in liquid N_2 and then homogenized in a household juicer with 200 mL of extraction buffer (0.2 M Tris, 10 mM EDTA, 5% [v/v] glycerol, 10 mM DTT, 0.1 mM E-64, 0.1 mM p-APMSF, and 5% [w/v] insoluble PVP, pH 8.0) per 100 g fresh weight. The homogenate was centrifuged for 30 min at 22,500*g*. Ammonium sulfate was added to the supernatant to yield a final concentration of 400 mM. After 1 h on ice, the precipitate was removed by centrifugation for 30 min at 22,500*g*.

The supernatant was loaded onto a TSK gel Butyl Toyopearl 650M column (10-mL bed volume for every 100 g fresh weight roots; Fractogel TSK Butyl-650M, Merck, Darmstadt, Germany) that had been equilibrated with a loading buffer (20 mM Tris, 1 mM EDTA, 3 mM DTT, 400 mM $(NH_4)_2SO_4$, and 0.1 mM p-APMSF, pH 8.0). NAS was eluted with elution buffer (10 mM Tris, 1 mM EDTA, 3 mM DTT, 0.1 mM p-APMSF, 5% glycerol, and 0.05% Chaps, pH 8.0).

KCl was added to the active fraction to give a final concentration of 0.4 M, and 1 M potassium phosphate buffer (pH 8.0) was added to a final concentration of 1 mM. The NAS fraction was loaded onto a hydroxyapatite column (100 to approximately 350 mesh; Nacalai Tesque, Kyoto, Japan; 10-mL bed volume for each 100 mg of protein in the NAS fraction) equilibrated with loading buffer (1 mM potassium phosphate buffer, 10 mM KCl, 3 mM DTT, and 0.1 mM p-APMSF, pH 8.0). NAS activity was eluted in the void volume.

The resulting NAS fraction was then loaded onto another TSK gel Butyl Toyopearl 650M column (1-mL bed volume for each 10 mg of protein in the NAS fraction) and eluted in the manner described above.

The active fraction was loaded onto a DEAE-Sepharose fast-flow column (5-mL bed volume for each 25 mg of protein in the NAS fraction, Pharmacia) equilibrated with the loading buffer (20 mM Tris, 1 mM EDTA, 3 mM DTT, and 0.1 mM p-APMSF, pH 8.0). The column was eluted using a stepwise gradient with increasing KCl concentration in the elution buffer (20 mM Tris, 1 mM EDTA, 3 mM DTT, 0.1 mM p-APMSF, and 0.05% Chaps, pH 8.0, with 50, 100, 150, or 200 mM KCl). The fraction with NAS activity was eluted with the buffer that contained 150 mM KCl.

The active fraction was loaded onto an Ether Toyopearl 650M column (0.5-mL bed volume; Fractogel TSK Butyl-650M, Merck) equilibrated with loading buffer (20 mM Tris, 1 mM EDTA, 3 mM DTT, 0.1 mM p-APMSF, and 1.2 M $[NH_4]_2SO_4$, pH 8.0). NAS did not bind to the column and was eluted in the void volume. This unbound fraction was subsequently loaded onto a Butyl Toyopearl 650M column (bed volume, 0.3 mL) equilibrated with loading buffer (20 mM Tris, 1 mM EDTA, 3 mM DTT, 0.1 mM p-APMSF, and 1.2 M $[NH_4]_2SO_4$, pH 8.0). The column was washed with buffer (20 mM Tris, 1 mM EDTA, 3 mM DTT, 0.4 M $[NH_4]_2SO_4$, and 0.1 mM p-APMSF, pH 8.0) and the NAS activity was then eluted with the elution buffer (10 mM Tris, 1 mM EDTA, 3 mM DTT, 0.1 mM p-APMSF, 5% glycerol, and 0.05% Chaps, pH 8.0).

The proteins in the active fraction were separated by SDS-PAGE at 4°C using 11% acrylamide slab gels. After SDS-PAGE the gel was stained with 0.3 M $CuCl_2$ (Dzandu et al., 1988), and then the stained bands were cut out. The gel fragments were destained with 0.25 M EDTA/0.25 M Tris (pH 9.0) and then homogenized with 1% SDS, 25 mM Tris, and 192 mM Gly. Each homogenate was placed in an elution cup and polypeptides were electroeluted with SDS-

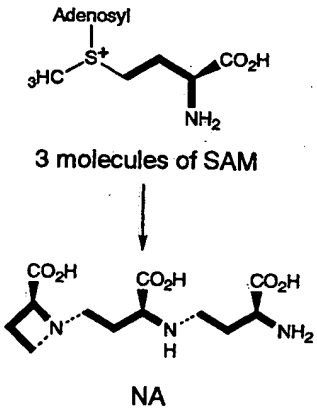

Figure 2. NAS catalyzes trimerization of SAM and ring formation to synthesize NA. Bold lines indicate the unit incorporated in NA. Hatched lines indicate newly formed N—C bonds.

free buffer containing 25 mM Tris and 192 mM Gly. The NAS fractions from all the steps described above were stored at 4°C with 10 μM E-64.

Gel Filtration

The active NAS fraction after hydroxyapatite chromatography was applied to a Sephacryl S300HR column (1.5 × 71 cm, 125 mL; Pharmacia) equilibrated with buffer (50 mM Tris, 1 mM EDTA, 100 mM KCl, 0.05% Chaps, 0.1 mM p-APMSF, and 3 mM DTT, pH 8.0). The column was calibrated with standard proteins: thyroglobulin (M_r 670,000), γ-globulin (M_r 158,000), ovalbumin (M_r 44,000), and myoglobin (M_r 17,000). Linear flow was 10 cm h^{-1}.

Determination of Partial Amino Acid Sequence

Proteins were digested with CNBr using the method of Gross (1967) with the following modifications. NAS peptide isolated on SDS-PAGE gels was eluted from the gel by homogenizing in a 10-fold volume of 70% (v/v) formic acid containing 1% (w/v) CNBr in a 1.5-mL tube and by subsequent overnight incubation at 4°C. The supernatant was collected, dried under a partial vacuum, resuspended in the SDS-PAGE sample buffer, and incubated overnight at room temperature. After the proteins were digested, the small peptides were separated by electrophoresis using Tricine SDS-PAGE (Schägger and Jagow, 1987) in 16.5% (w/v) acrylamide gels. The peptides were transferred onto a PVDF membrane by electroblotting (Towbin et al., 1979) and stained with amido black. Each band on the PVDF membrane was cut out and the amino acid sequence was determined by automated Edman degradation in a gas-phase sequencer (model 492A protein sequencer, model 785A programmable absorbance detector, and model 140C microgradient system, Applied Biosystems).

Cloning of *nas* Genes

A pYH23 cDNA library prepared from the poly(A^+) RNA of Fe-deficient barley roots was screened with a PCR product corresponding to the sequence of the NAS homolog of rice, expressed sequence tag cDNA clone RICR2562A (D24790), and RICR0168A (D23792). The primers used were: 5′-ATGGAGGCTCAGAACCAAGAGGTCGC-3′ (N-terminal forward primer) and 5′-GGATGAGCTCCTCCCGCATCGCCT - 3′ (N - terminal reverse primer) from D24790, and 5′-CAACCTGAGCAAGCTGGAGTACGACC-3′ (internal forward primer) and 5′-TTCTTCTCGGCCGCCATGCCCACGA-3′ (internal reverse primer) from D23792 (Fig. 5). The probe obtained by PCR amplification was labeled with [α-^{32}P]dATP using the random primer-labeling kit version 2 (TaKaRa, Shiga, Japan). The labeled DNA was purified in a ProbeQuant G-50 microcolumn (Pharmacia). The isolated cDNA clones were sequenced by the Thermo Sequenase Cycle Sequencing Kit (Shimadzu, Tokyo, Japan), following the protocol of the manufacturer, using a DNA sequencer (model DSQ-1000L, Shimadzu).

Expression in *Escherichia coli*

An *Eco*RI site was introduced near the first ATG of the *nas1* cDNA, and a *Pst*I site was introduced near the first stop codon of the *nas1* cDNA by PCR mutagenesis. The primers used were 5′-GAGAGAGAGAATTCGCCATGGATGCCCAGAACAAGGAG-3′ and 5′-GAGAGAGAGGATCCCTGCAGCTTCAATCAAAAGGCCAGCTC-3′ (*Eco*RI and *Pst*I sites are underlined). An *Eco*RI-*Pst*I fragment containing the *nas1*-coding sequence was excised from the PCR product and cloned into pMAL-c2 (New England Biolabs) to give pMAL-NAS1, which was introduced into *E. coli* XL1-Blue, and the recombinant bacteria were cultured in Luria-Bertani medium containing 100 μg mL^{-1} ampicilllin and 20 μg mL^{-1} tetracyclin at 37°C until the A_{600} of the culture reached 0.5. At this time isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.3 mM. After 4 h a crude extract from the cells was prepared as described by the manufacturer of the pMAL kit.

Northern-Blot Analysis

The hybridization probe used for northern blots was a *Hin*dIII-*Not*I restriction fragment containing the full-length *nas1* cDNA labeled with [α-^{32}P]dATP. The labeled DNA was purified in a ProbeQuant G-50 microcolumn (Pharmacia). Total RNA was isolated from the roots or leaves of barley according to the procedure of Naito et al. (1988). RNA (5 μg per lane) was separated on 1.4% (w/v) agarose gels containing 5% (v/v) formaldehyde and blotted onto membranes (Hybond-N^+, Amersham). The membrane was hybridized with the labeled probe in 0.5 M Church phosphate buffer (Church and Gilbert, 1984), 1 mM EDTA, and 7% (w/v) SDS with 100 μg mL^{-1} salmon sperm DNA at 65°C overnight. After hybridization the blot was washed twice with 40 mM Church phosphate buffer and 1% (w/v) SDS at 65°C for 10 min and at high stringency with 2× SSPE and 0.1% (w/v) SDS at 65°C for 10 min, and radioactivity was then detected using a BAS-2000 image analyzer (Fuji, Tokyo, Japan).

Southern-Blot Analysis

Genomic DNA prepared from leaves of barley and rice was digested with *Bam*HI, *Eco*RI, or *Hin*dIII, separated on a 0.8% (w/v) agarose gel (10 μg per lane), and alkali transferred onto a Hybond-N^+ membrane. The membrane was hybridized with the same probes under the same conditions as described above.

RESULTS

Improvement of the Extraction and Column Chromatography Procedure Used to Purify NAS from Fe-Deficient Barley Roots

We previously reported the partial purification of NAS (Higuchi et al., 1994), but we were unable to obtain sufficient amounts of protein to determine its partial amino acid

sequence. Subsequently, we discovered that E-64, a thiol protease inhibitor, was very effective in protecting NAS from degradation (Higuchi et al., 1996a). In this study frozen roots were crushed to a fine powder in liquid N_2 and then rapidly homogenized with buffer containing 0.1 mM E-64 to avoid degradation of NAS. This improvement increased the recovery of NAS about 20-fold.

In the previous work NAS activity was detected in the 30- to 35-kD protein fraction recovered from the SDS-polyacrylamide gel after the removal of SDS (Higuchi et al., 1994), but the rate of recovery was very low. Therefore, we further improved the column chromatography procedures. NAS is relatively hydrophobic and a buffer containing Chaps effectively increased the rate of recovery and the resolution of the column chromatography. Several ion-exchange chromatography media were tested, and DEAE-Sepharose fast-flow and DEAE Sephacel were found to be the most effective. Both Butyl Toyopearl and another hydrophobic chromatography medium, Ether Toyopearl, effectively removed impurities from the 30- to 35-kD fraction.

Comparison of the Peptides on SDS-PAGE in the Purified NAS Fraction from Fe-Deficient Barley Roots and Control Barley Roots

In general, NAS activity was detected as a broad peak on the SDS-polyacrylamide gel from 30 to 35 kD. To identify the NAS protein, we compared the peptides in the NAS fraction obtained from Fe-deficient barley roots with those from Fe-sufficient (control) barley roots. NAS was purified from 200 g of Fe-deficient and control roots. The NAS activity of the control roots was about one-quarter of the Fe-deficient roots at each purification step. The active NAS fraction from each purification step was analyzed by SDS-PAGE (Fig. 3). An almost identical pattern was observed in both Fe-deficient and control roots before the DEAE-Sepharose step (Fig. 3A). After the DEAE-Sepharose step it became clear that the 30- and 31-kD peptides were induced by Fe deficiency (Fig. 3B). After the Ether Toyopearl step, the 31-kD peptide was eliminated from the active NAS fraction. Two more Fe-deficiency-inducible peptides that were 32 and 33 kD in size were also detected in the active NAS fraction (Fig. 3C). Both the 32- and 33-kD peptides had NAS activity, but the 30-kD peptide was inactive (Fig. 4).

Estimation of the M_r of NAS Using Gel Filtration

According to previous data, the M_r of NAS is between 40,000 and 50,000 (Higuchi et al., 1994), but this did not correspond with the value estimated by SDS-PAGE. In this study the buffer containing Chaps effectively increased the recovery of active enzyme and the resolution of the peak pattern of the column. Consequently, the M_r of NAS was more accurately determined to be 35,000 (Fig. 5) and corresponds well to the value estimated by SDS-PAGE.

Determination of the Partial Amino Acid Sequence of NAS

The active NAS fraction was purified from 1 kg of Fe-deficient barley roots. Because 32- and 33-kD peptides from the preparative SDS-PAGE gel could not be completely separated from each other, they were digested together with CNBr, whereas the 30-kD peptide was digested separately. The partial amino acid sequences of the fragments obtained from the 32- plus 33-kD peptides and the 30-kD peptide were homologous to each other (Fig. 6). The M_r of the 33- plus 32-kD-1 fragment (Fig. 6) was almost the same as the original; thus, we speculated that this sequence corresponded to the N-terminal region of NAS. A search of the database revealed that these putative NAS amino acid

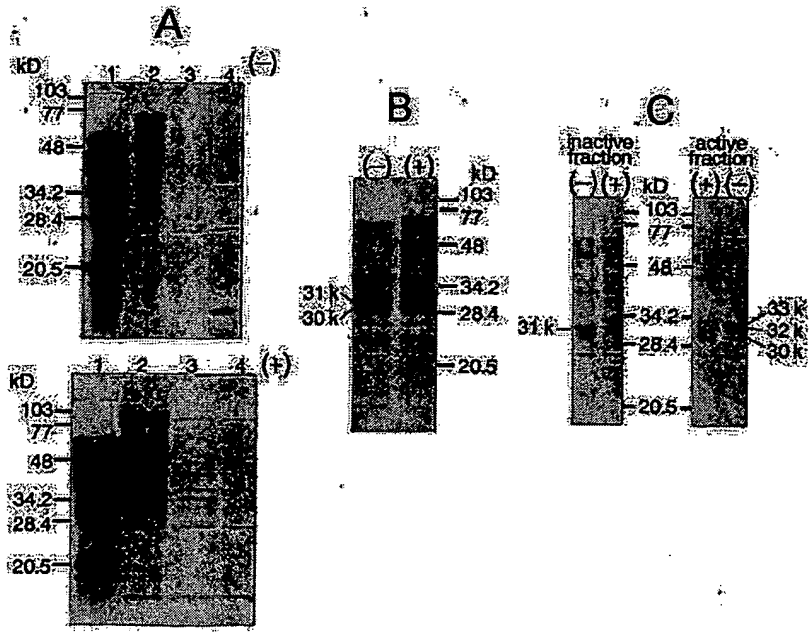

Figure 3. Comparison of NAS purification from Fe-deficient (−) and Fe-sufficient (control, +) barley roots. SDS-PAGE was carried out using 12.5% acrylamide slab gels (Laemmli, 1970). Gels were stained with Coomassie brilliant blue. A, top, From Fe-deficient barley roots; bottom, from control barley roots. Lanes 1, Crude extract, 200 μg of protein. Lanes 2, After Butyl Toyopearl 650M, 100 μg of protein. Lanes 3, After hydroxyapatite, 20 μg of protein. Lanes 4, After Butyl Toyopearl 650M, 15 μg of protein. B, After DEAE-Sepharose fast flow, 25 μg of protein. C, After Ether Toyopearl 650M, one-quarter of each fraction.

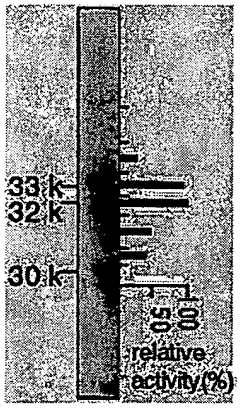

Figure 4. Preparative SDS-PAGE was carried out using 11% acrylamide slab gels. A portion of the gel in this figure was stained with Coomassie brilliant blue and the rest of the gel was stained with Cu. The gel containing proteins between 30 and 35 kD in size was cut into seven fragments (indicated by the short lines). The recovery was low but NAS activity was reproducibly detected by elimination of SDS and Cu during electroelution of peptide from the gels. The thick bars indicate relative NAS activity of peptides from each gel fragment. The activity of the most active fraction was set at 100%.

sequences were similar to the translation products of rice expressed sequence tag cDNA clones of unknown function (accession nos. D23792 and D24790), with 80.0% identity in a 33-amino acid overlap in the former and 68.4% identity in a 19-amino acid overlap in the latter (Fig. 6).

Cloning and Nucleotide Sequences of cDNA Clones Encoding NAS

PCR amplification of total cDNA prepared from Fe-deficient barley roots using degenerate primers designed from the partial amino acid sequence of barley NAS was not successful. We then used partial sequences from the rice expressed sequence tag clones (Fig. 6, arrows) as the primers. The resulting 205-bp amplification product using N-terminal forward and N-terminal reverse primers and a 274-bp amplification product using internal forward and internal reverse primers were used as probes for hybridization. A cDNA library prepared using poly(A^+) RNA from Fe-deficient barley roots was screened and 19 positive clones using the 205-bp probe and 88 positive clones using the 274-bp probe were obtained.

Nucleotide sequence analysis of one of these positive clones, designated *nas1*, revealed an open reading frame of 985 bp encoding a 328-amino acid polypeptide with a predicted M_r of 35,144. This corresponded well with the size of the NAS polypeptide estimated by SDS-PAGE. The partial amino acid sequences of the 32- plus 33-kD peptide matched the portions of the deduced amino acid sequence of *nas1* (Fig. 7, underlined). The predicted pI of 5.2 matched the value estimated by native IEF electrophoresis well (data not shown). Another six NAS-like clones, *nas2*, *nas3*, *nas4*, *nas5-1*, *nas5-2*, and *nas6*, were also obtained (Table I; Fig. 8). The nucleotide sequences for the NAS genes from barley have been deposited in the database and given the following accession numbers: AB010086 (*nas1*), AB011265 (*nas2*), AB011264 (*nas3*), AB011266 (*nas4*), AB011267 (*nas5-1*), AB011268 (*nas5-2*), and AB011269 (*nas6*). The partial amino acid sequences of the 30-kD peptide matched portions of the deduced amino acid sequence of *nas5-1* and *nas5-2* (Fig. 8, underlined). The 5′ and 3′ noncoding region of these seven clones differed from each other, except *nas5-1* and *nas5-2*. Sequences of *nas5-1* and *nas 5-2* were identical, with the exception of the 122 to 140 amino acid residues of *nas5-2*. *nas5-1* may be a deletion clone of *nas5-2*, although we did not determine whether this deletion clone is an artifact.

Expression of NAS1 in *E. coli*

To confirm the enzymatic function of the gene product, *nas1* was expressed as a maltose-binding protein fusion in *E. coli*. The bacterial strains containing the NAS1 expression vector or the empty vector were induced with isopropyl β-D-thiogalactopyranoside, and the crude extracts were analyzed for NAS activity by TLC (Fig. 9). The crude extract from the strain transformed with pMAL-NAS1 had NAS activity, whereas the crude extract from the strain transformed with the vector had no NAS activity.

Northern-Blot Analysis

Northern hybridization analysis revealed that *nas1*-mRNA was not detected in either the leaves or roots of the control plants. In contrast, *nas1* was highly expressed in the roots but not in the leaves of Fe-deficient plants (Fig. 10). This result corresponds well with the previously described expression pattern of NAS activity (Higuchi et al., 1994).

Southern-Blot Analysis

The number of *nas* and *nas*-like genes in the barley and rice genomes was assessed by Southern-blot analysis (Fig.

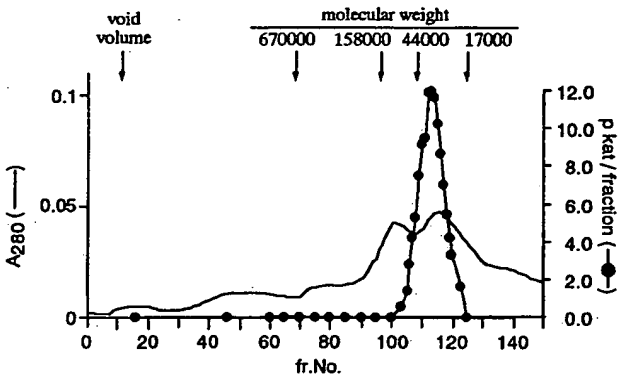

Figure 5. Elution pattern of NAS activity from the gel-filtration column. The black circles indicate NAS activity. The solid line indicates protein concentration monitored at 280 nm. The active NAS fraction after hydroxyapatite chromatography was applied to a Sephacryl S300HR column (1.5 × 71 cm, 125 mL). The column was calibrated with standard proteins: thyroglobulin (M_r 670,000), γ-globulin (M_r 158,000), ovalbumin (M_r 44,000), and myoglobin (M_r 17,000). The linear flow was 10 cm h^{-1}.

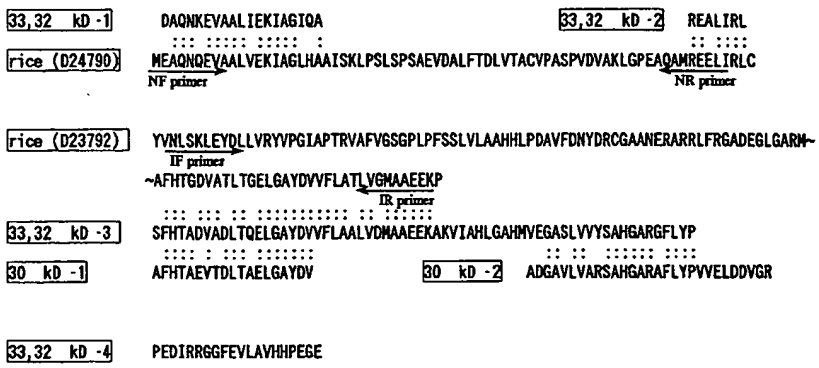

Figure 6. Partial amino acid sequence of combined 32- plus 33-kD peptides and that of the 30-kD peptide. The amino acid sequences of two rice homologs are also shown. The arrows indicate PCR primers (for the DNA sequences of primers, see "Materials and Methods"). IF, Internal forward primer; IR, internal reverse primer; NF, N-terminal forward primer; NR, N-terminal reverse primer.

11). With the barley genome DNA, cutting with *Bam*HI and *Eco*RI each produced 7 bands, which were detected on Southern blots, whereas cutting with *Hin*dIII produced 10 bands. None of the seven clones obtained in this work have *Bam*HI or *Eco*RI sites, and only *nas2* and *nas4* have *Hin*dIII sites. This result corresponds well with the existence of at least seven *nas* cDNA clones.

DISCUSSION

In this paper we describe the partial purification of several NAS proteins induced by Fe deficiency (Fig. 4) and their corresponding genes (Fig. 8). The enzymatic function of the *nas1* product was confirmed (Fig. 9). In addition, a 30-kD peptide and its gene, *nas5-1* or *nas5-2*, which are homologous to NAS but lack NAS activity, were also found (Fig. 4). Several NAS proteins and their corresponding genes were found, but it remains unclear whether each NAS protein functions differently. It is possible that a heteropolymer composed of these homologous 30-, 32-, and 33-kD peptides forms the native enzyme. However, the M_r of NAS estimated by gel filtration was 35,000 (Fig. 5), and we did not observe increased NAS activity by combining the 30-, 32-, and 33-kD peptides in vitro (data not shown). Therefore, we tentatively conclude that native NAS exists as a monomer.

Another possibility is that native NAS exists as a complex of 30-, 32-, and 33-kD peptides, because NAS proteins are relatively hydrophobic, indicating that they may be membrane bound and localized in some organelle (Fig. 12). This may allow the efficient catalysis of trimerization of SAM. We have already shown that radioactivity from [1-^{14}C]Met, the precursor of MAs, is localized in "particular vesicles" in the Fe-deficient barley root cell (Nishizawa and Mori, 1987; Nishizawa et al., 1990). This suggests that biosynthesis of MAs occurs inside particular vesicles.

The catalytic mechanism of NA synthesis from SAM by NAS may be similar to that of methyl transferase using SAM as a methyl donor or those of spermidine synthase and spermine synthase using decarboxy-SAM as a substrate (Pajula et al., 1979). The common catalytic domain of these enzymes has been discussed in relation to amino acids occupying similar positions in the secondary and tertiary structures (Schluckebier et al., 1995; Hashimoto et al., 1998). If the 30-kD peptide lacking NAS activity has SAM-binding activity, comparison of NAS protein and the inactive NAS homolog may reveal the catalytic or the binding domain structure of NAS. Because NA is contained in all plants surveyed so far, NAS may be widespread among plant species. The translation products of the putative rice NAS homologs, D23792 and D24790, were very

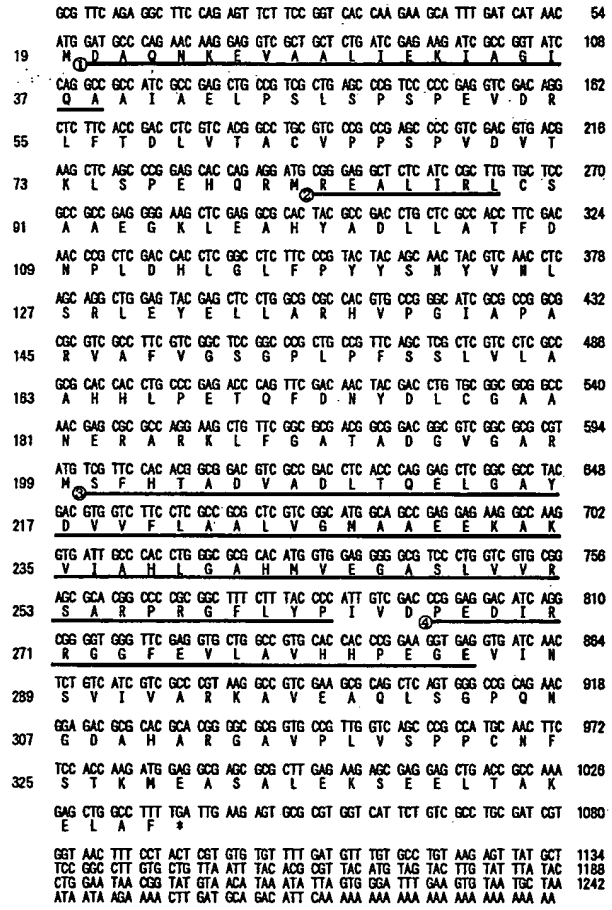

Figure 7. Nucleotide and deduced amino acid sequence of the barley *nas1* cDNA clone. The underlined sequences indicate four partial amino acid sequences of fragments from combined 32- plus 33-kD peptides. The nucleotide sequence is numbered from the 5′ end of the cDNA clone and indicated to the right of each row. Amino acid numbers are indicated on the left of each row.

Table I. *Properties of deduced peptides from nas clones*
pI values were predicted by the DNASIS program (Hitachi, Tokyo, Japan).

Clone	Amino Acid Residues	M_r	pI	Identity to *nas1*	Identity to *nas2*	Identity to *nas4*
	no.				%	
nas1	328	35,144	5.20	—		
nas2	336	35,839	5.07	72	—	
nas3	336	36,013	5.47	72	95	
nas4	330	35,396	4.91	73	89	—
nas5-1	268	28,802	5.32	57	58	56
nas5-2	283	30,148	5.22	61	61	59
nas6	329	35,350	5.07	74	89	88

similar to NAS1 protein, with 80.2% identity in a 111-amino acid overlap in the former and 78.9% identity in a 71-amino acid overlap in the latter. Putative Arabidopsis NAS homologs, AC003114 (function unknown) and AB005245 (function unknown), were also found with 45% identity in the former and 46% identity in the latter to NAS1. Therefore, comparison of NAS protein homology among many species may also reveal information about its catalytic domain.

Since full-length *nas1* used as a probe could also detect other *nas* genes, a 1.3-kb band smear was seen on northern blots (Fig. 10), and many bands were detected on Southern blots (Fig. 11). Therefore, we conclude that *nas* belongs to a multigene family in the barley genome and probably also in the rice genome. The number of copies should be confirmed by Southern-blot analysis using conserved sequences of *nas* as the probes.

On northern blots a smeared band produced the strongest signal, but weak and short bands were also detected. These may represent degradation products of *nas1* mRNA (Fig. 10). Since NAS proteins are vulnerable to degradation, it is quite possible that the expression of the *nas* gene in barley roots is strictly controlled to avoid overproduction of MAs. For instance, resupplying Fe to Fe-deficient barley decreased NAS activity within 24 h (Higuchi et al., 1996a).

We could tentatively categorize the seven *nas* clones into several types based on homology of amino acid sequences:

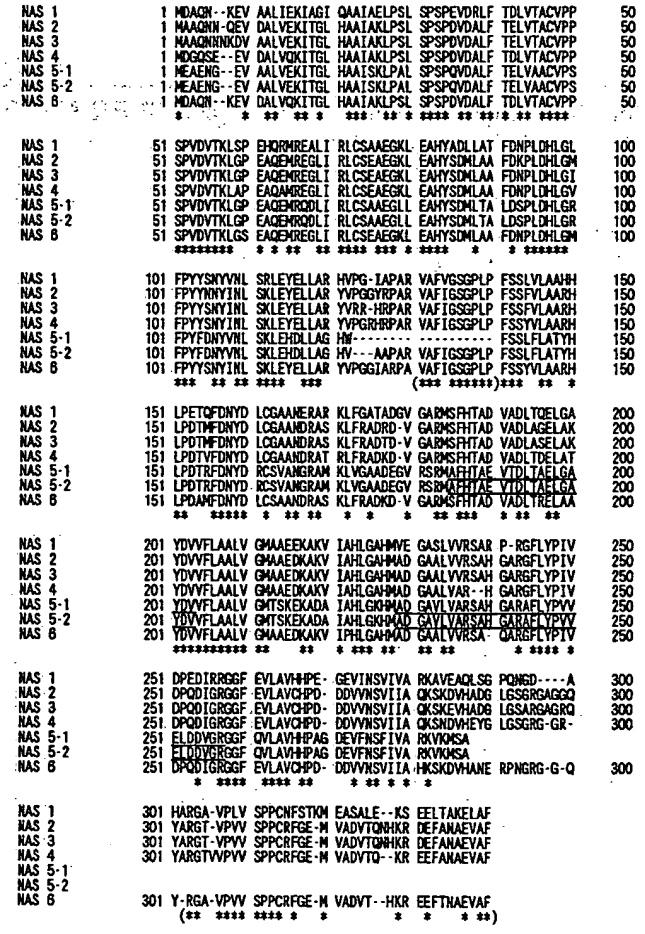

Figure 8. Comparison of the deduced amino acid sequences of barley proteins NAS 1 to NAS 6. Asterisks indicate identical amino acid residues in all sequences. Asterisks in parentheses indicate identical amino acid residues in all sequences except NAS 5-1 and 5-2. The underlined sequences indicate the partial amino acid sequences of fragments from 30-kD peptide.

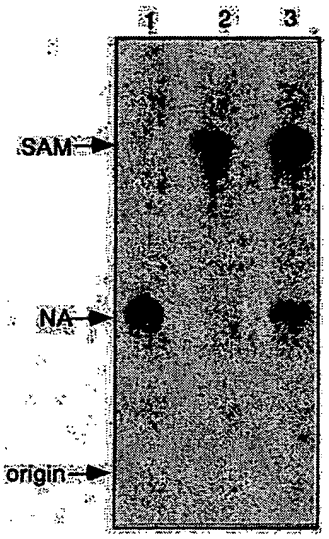

Figure 9. TLC analysis of NAS activity assay mixture from *E. coli* expressing NAS1. Lane 1, Standard NA; lane 2, pMAL only; lane 3, pMAL-NAS1.

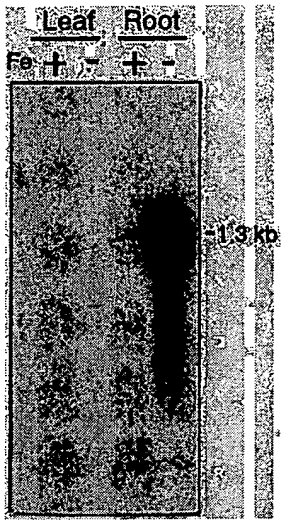

Figure 10. Northern-blot analysis of *nas1*. RNA was extracted from Fe-deficient (−) and control (+) roots and leaves. Each lane was loaded with 5 μg of RNA. Total RNA was extracted after 1 week of Fe-deficiency treatment.

type 1, *nas1*; type 2, *nas2*, 3, 4, and 6; and type 3, *nas5* (Table I). Nineteen independent *nas1* clones were obtained from a cDNA library prepared using poly(A^+) RNA from Fe-deficient barley roots, but only one *nas4* clone was obtained. The numbers of clones of *nas2*, *nas3*, *nas5-1*, *nas5-2*, and *nas6* were two, four, five, three, and three, respectively.

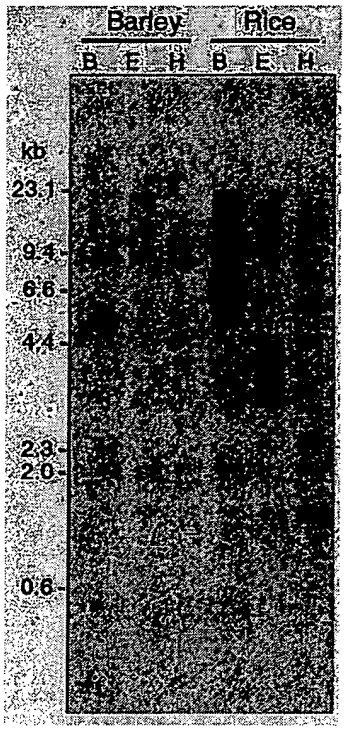

Figure 11. Southern-blot analysis of *nas*-like genes. Genomic DNA from barley and rice were digested with *Bam*HI (lanes B), *Eco*RI (lanes E), and *Hin*dIII (lanes H) and probed with *nas1* under high-stringency conditions.

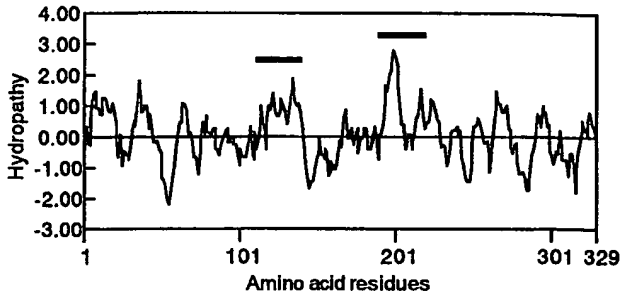

Figure 12. Hydropathy profile of NAS1. Hydrophobicity was analyzed by the DNASIS program, as described by Kyte and Doolittle (1982), with a window size of 10 amino acid residues. Hydrophilic domains are below the zero line. Bold lines indicate hydrophobic regions.

These results suggest that the expression of *nas* genes under Fe deficiency differs from one *nas* gene family to the next. Northern-blot analysis using a specific probe for each *nas* gene will reveal the precise expression pattern of each gene and suggest the function of each *nas* gene under different stress conditions.

Until now, NA, the reaction product of NAS, has been thought to function only as an intermediate substrate for MAs synthesis in graminaceous plants (Fig. 1). Since the *nas* gene belongs to a multigene family, NA has a function in the survival of graminaceous plants other than as a precursor of MAs. In Strategy I plants, which lack the ability to produce MAs, it has been proposed that NA plays a key role as an endogenous chelator of divalent metal cations, such as Fe^{2+}, Cu^{2+}, Zn^{2+}, and Mn^{2+}, in the xylem and phloem and that it contributes to the homeostasis of those metals in plants (Stephan et al., 1994; Higuchi et al., 1996b). NA may also play a role in the metabolism of Fe or other metals in Strategy II plants. The fact that NAS activity is not enhanced by Fe deficiency in Strategy I plants (Higuchi et al., 1995, 1996b) suggests that expression of the *nas* gene is not regulated by Fe in these plants. Transgenic plants with suppressed NAS expression will help clarify the role of NA in both Strategy I and Strategy II plants.

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Presence of Nicotianamine Synthase Isozymes and Their Homologues in the Root of Graminaceous Plants

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Nicotianamine synthase (NAS) catalyzes the synthesis of nicotianamine, which is an intermediate in the biosynthetic pathway of mugineic acid family phytosiderophores (MAs). Using polyclonal anti-NAS antibodies and recombinant NAS proteins, we identified five NAS isozymes and one NAS homologue in Fe-deficient barley roots using two-dimensional electrophoresis followed by Western blot analysis. Other unidentified NAS homologues that were induced by Fe-deficiency were also detected in barley roots. Western analysis enabled to detect NAS homologues in wheat, oats, rice, maize, and sorghum roots. In graminaceous species, both the amount and number of NAS homologues were correlated with the total NAS activity and Fe-deficiency tolerance. The NAS isoform patterns differed among the graminaceous plants.

Key Words: **Fe-deficiency, graminaceous plant, mugineic acid, nicotianamine synthase, Western blot analysis.**

Graminaceous plants secrete iron-chelators, called mugineic acid-family phytosiderophores (MAs), from their roots to solubilize sparingly soluble iron in the rhizosphere. MAs is the only class of phytosiderophores so far identified in plants (Takagi 1976). The amount of phytosiderophores secreted increases dramatically under Fe-deficiency stress. In graminaceous plants, tolerance to Fe-deficiency is considered to depend on the amount of MAs plant roots secrete under Fe-deficiency stress (Takagi et al. 1984; Marschner et al. 1987; Singh et al. 1993).

Increase in the activity of nicotianamine synthase (NAS) and nicotianamine aminotransferase (NAAT) is essential to the enhancement of MAs biosynthesis in Fe-deficient plants and therefore to tolerance to Fe-deficiency (Kanazawa et al. 1994; Higuchi et al. 1996a). Recently, we have purified NAS and NAS-like proteins from Fe-deficient barley roots and isolated 7 related cDNA clones of NAS from barley (Higuchi et al. 1999). In this study, we detected a number of NAS-like proteins in barley and in five other graminaceous species by Western blot analysis. The correlations between the amount of NAS-like protein or its isoform patterns with NAS activity are discussed.

MATERIALS AND METHODS

Preparation of polyclonal antibodies to NAS. Two mice were immunized with a total of 100 μg NAS peptides which were the same as those used to determine the partial amino acid sequences described in the report of Higuchi et al. (1999). For the first injection, the immunogen was emulsified in complete Freund's adjuvant. For the second and subsequent injections, incomplete Freund's adjuvant was used. After the 4th induction, whole blood was collected and the antiserum was stored at −80°C until use.

Western blot analysis. The procedure described by Damerval et al. (1986) using trichloroacetic acid (TCA) and acetone extraction of proteins was applied with slight modifications. The plant materials were crushed in liquid N_2 using a mortar and pestle, and the powder was then resuspended in a cold solution of 100 g L^{-1} TCA in acetone with 1 mg g^{-1} 2-mercaptoethanol (2-ME). Proteins were allowed to precipitate for 1 h at −20°C, and then centrifuged at 16,000×*g* for 30 min at 4°C. The supernatant solution was discarded and the pellet containing both proteins and residues was rinsed with cold acetone containing 1 mg g^{-1} 2-ME for 1 h at −20°C, and then centrifuged at 16,000×*g* for 30 min at 4°C. The supernatant solution was discarded, the pellet was dried under reduced pressure, then the proteins were dissolved with sample buffer (50 μL mg^{-1} dry pellet: 9.5 M urea, 20 g L^{-1} Triton X-100, 50 mg g^{-1} 2-ME). The suspension was centrifuged at 16,000×*g* for 10 min at room temperature, then the residues were removed. The supernatant solution was applied to SDS-PAGE (Laemmli 1970) or two-dimensional electrophoresis (2D-PAGE) (O'Farrell 1975). The peptides were transferred onto a PVDF membrane by electroblotting and used for Western analysis, which was performed using the NAS antibodies described above with a secondary antibody that was a conjugate of goat anti-mouse IgG (H+L) and horseradish peroxidase (Wako, Osaka, Japan). The blot was stained with diaminobenzidine.

Expression of recombinant NAS proteins in *Escherichia coli*. PCR mutagenesis was used to introduce *Eco*RI and *Nco*I sites close to the first ATG into the seven *nas* cDNAs (Higuchi et al. 1999). Restriction sites were also introduced near the first stop codon of the seven *nas* cDNAs. *Pst*I and *Bam*HI sites were introduced into *hvnas1, hvnas2, hvnas3*, and *hvnas6*, a *Bam*HI site into *hvnas4*, and *Hin*dIII and *Bam*HI sites into *hvnas5-1* and *hvnas5-2*. The following primers were used for the N-terminal regions (the *Eco*RI and *Nco*I sites are underlined).

For *hvnas1, hvnas4*, and *hvnas6*:

5′-GAGAGAGAGAATTCGCCATGGATGCCCAGAACAAGGAG-3′.

For *hvnas2* and *hvnas3*:

5′-GAGAGAGAGAATTCGCCATGGCTGCCCAGAACAAC-3′.

For *hvnas5-1* and *hvnas5-2*:

5′-GAGAGAGAGAATTCGCCATGGAGGCCGAAAACGGCGAG-3′.

The following primers were used for the C-terminal regions (the *Pst*I, *Hin*dIII, and *Bam*HI sites are underlined).

For *hvnas1*:

5′-GAGAGAGAGGATCCCTGCAGCTTCAATCAAAAGGCCAGCTC-3′ (*Pst*I and *Bam*HI sites).

For *hvnas2, hvnas3*, and *hvnas6*:

5′-GAGAGAGAGGATCCCTGCAGCGATCAAAAGGCCACTTCGGC-3′ (*Pst*I and *Bam*HI sites).

For *hvnas4*:
5′-GAGAGAGAGGATCCCTCGAGCAATCAGAAGGCCACTTCCGC-3′ (*Bam*HI site).

For *hvnas5-1* and *hvnas5-2*:
5′-GAGAGAGAGGATCCAAGCTTAATTTAAGCACTCATTTTCAC-3′ (*Hin*dIII and *Bam*HI sites).

The appropriate restriction fragments containing the coding sequences of the seven cDNAs were excised from the PCR products and cloned into pMAL-c2 (New England Biolabs) to give pMAL-NAS, which were introduced into *E. coli* XL1-Blue. The recombinant bacteria were cultured in Luria-Bertani (LB) medium containing 100 μg mL^{-1} ampicillin and 20 μg mL^{-1} tetracycline at 37°C until the OD_{600} of the culture medium reached a value of 0.5. At this time, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.3 mM to induce the production of the recombinant protein. After 4 h, a crude extract from the cells was prepared, and recombinant fusion proteins were purified using 'Amylose Resin' as described in the manufacturer's manual for the pMAL kit.

*Nco*I-*Bam*HI fragments containing the coding sequences of *hvnas1*, *hvnas2*, *hvnas3*, *hvnas4*, *hvnas5-2*, and *hvnas6* were excised from the PCR products and cloned into pET-16b (Novagen) to give pET-NAS, which were introduced into *E. coli* BL21 (DE3). The recombinant bacteria were cultured in LB medium containing 100 μg mL^{-1} ampicillin at 37°C until the OD_{600} of the culture medium reached a value of 0.5. At this time, IPTG was added to a final concentration of 0.3 mM to induce the production of the recombinant protein. After 4 h, the cells were harvested and suspended in SDS-PAGE sample buffer (O'Farrell 1975).

Assay of NAS activity. Enzyme solutions were equilibrated with reaction buffer (50 mM Tris, 1 mM EDTA, 3 mM dithiothreitol, 10 μM (*p*-amidinophenyl)methanesulfonyl fluoride (*p*-APMSF), 10 μM trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), pH 8.7) and concentrated by ultrafiltration using Ultrafree C3LGC NMWL10000 (Millipore Co.). The details of the method used to detect NAS activity were described in the report of Higuchi et al. (1996a).

Identification of six *nas* gene products on 2D-PAGE gels. Recombinant NAS proteins expressed using pET-NAS were isolated by SDS-PAGE and electroelution. Each recovered NAS was mixed with sample buffer (9.5 M urea, 20 g L^{-1} Triton X-100, 50 mg g^{-1} 2-ME) and applied to 2D-PAGE.

Plant materials. Barley (*Hordeum vulgare* L. cv. Ehimehadaka no. 1), oats (*Avena sativa* L. cv. Yakushin), wheat (*Triticum aestivum* L. cv. Arona), maize (*Zea mays* L. cv. Alice), sorghum (*Sorghum bicolor* (L.) Moench. cv. Big Jim), and rice (*Oryza sativa* L. cv. Nihonbare) were cultured as previously described (Higuchi et al. 1996a).

NAS activity in roots. Each aliquot of 0.5 g of frozen root tissue was crushed into a fine powder in liquid N_2 using a mortar and pestle, homogenized with extraction buffer, and then the NAS activity of the crude extract was detected following the method of Higuchi et al. (1999).

RESULTS AND DISCUSSION

Preparation of anti-NAS polyclonal antibodies

Polyclonal antibodies were raised against the 33 plus 32 kDa peptides, which consisted of the mixture of NAS isozymes used to determine the partial amino acid sequences of NAS

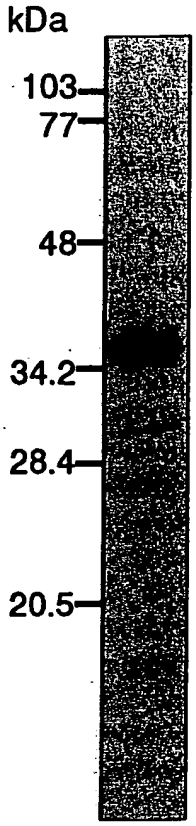

Fig. 1. Western blot analysis of total protein extracted from Fe-deficient barley roots with TCA/acetone. 40 μg of protein was loaded on the gel. 125 g L^{-1} acrylamide slab gels were used for SDS-PAGE. A 10^{-4} dilution of the antisera was used to detect NAS or NAS-like proteins.

(Higuchi et al. 1999). SDS-PAGE followed by Western blot analysis of total protein extracted from Fe-deficient barley roots by the TCA/acetone method indicated that a 10^{-4} dilution of the antisera specifically recognized NAS, and the molecular weight of the NAS band was 35–36 kDa (Fig. 1). This value matched well the molecular weights deduced from the sequences of the nas cDNAs (Higuchi et al. 1999), because NAS proteins extracted by the TCA/acetone method were intact. The 33 plus 32 kDa peptides as the antigen were extracted and purified under native conditions, thus NAS was partially degraded by some proteases (Higuchi et al. 1999).

NAS and NAS-like proteins in barley

More than seven protein spots were detected in extracts from Fe-deficient barley roots by 2D-PAGE followed by Western blot analysis (Fig. 2). All the proteins were induced by Fe-deficiency in roots. These results coincide with the presence of a number of NAS genes that were induced by Fe-deficiency (Higuchi et al. 1999). The pI values of these spots (5.0–5.5) also matched well those deduced from the *nas* cDNA sequences.

The molecular weights of most of the protein spots were 35–36 kDa, and a few spots showed a molecular weight of about 30 kDa. The deduced molecular weights of *hvnas1–hvnas4* and *hvnas6* proteins were 35–36 kDa, and those of *hvnas5-1* and *hvnas5-2* proteins were 28.2 and 30.1 kDa, respectively. *hvnas5-1* is a deletion clone of *hvnas5-2* (Higuchi et al. 1999). Since no NAS-like proteins under 30 kDa were detected in the Western blot analysis, *hvnas5-1* was considered to be an artifact produced during the preparation of the cDNA library.

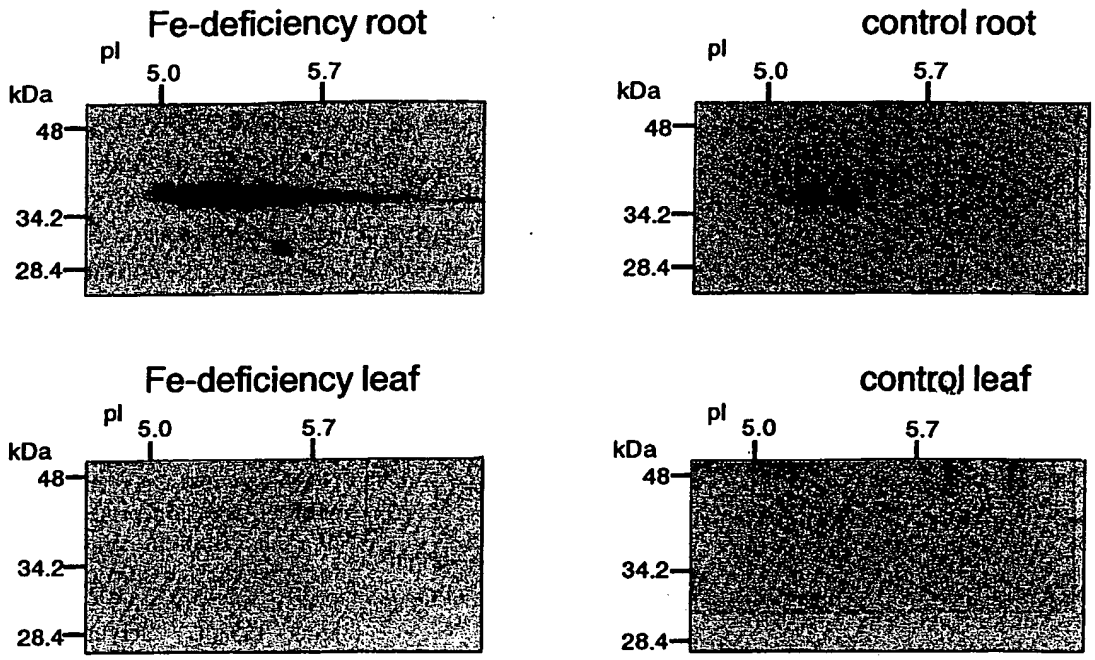

Fig. 2. Western blot analysis of total protein extracted from barley with TCA/acetone. 125 g L^{-1} acrylamide slab gels were used for 2D-PAGE. Either 200 μg of root protein or 500 μg of leaf protein was loaded on the gel.

In both control leaf and Fe-deficient leaf, few spots were observed which was in agreement with the fact that no signals were detected in leaves by Northern analysis (Higuchi et al. 1999). In the case of barley, MAs may be synthesized mainly in roots. Even if nicotianamine (NA) also plays a key role as an endogenous chelator of divalent metal cations (Stephan et al. 1994) in leaf, the amount of NA required may be lower than that for MAs-synthesis in the roots.

Since the deduced amino acid sequences of *hvnas1-hvnas6* were homologous to each other with 57–95% identity (Higuchi et al. 1999), we considered that the antisera recognized a wide range of NAS and NAS-like proteins.

NAS activity of *nas* gene products

Since all the gene products of the seven related *nas* clones were assumed to be present among the proteins detected by Western blot analysis, we tried to confirm the enzymatic function of each protein and to identify each protein individually on the 2D-PAGE gel.

Higuchi et al. (1999) previously confirmed the enzymatic function of the *hvnas1* gene product. To confirm the enzymatic functions of the other *nas*-like gene (*hvnas2, 3, 4, 5-1, 5-2*, and *6*) products, these genes were expressed as maltose-binding protein-fusions (MBP-NASs) in *E. coli*. MBP-NASs were purified using Amylose Resin affinity columns, and 0.5 μg of purified MBP-NAS was used for each enzyme assay (Fig. 3). MBP-HvNAS1, 2, 3, 4, and 6 showed a NAS activity, while MBP-HvNAS5-1 and 5-2 did not (Fig. 3A), even when 5 μg was used (data not shown). When the protein with the *hvnas5* sequence was purified from barley roots, it lacked the NAS activity (Higuchi et al. 1999).

The same 0.5 μg MBP-NAS samples used to confirm the NAS activity were analyzed by

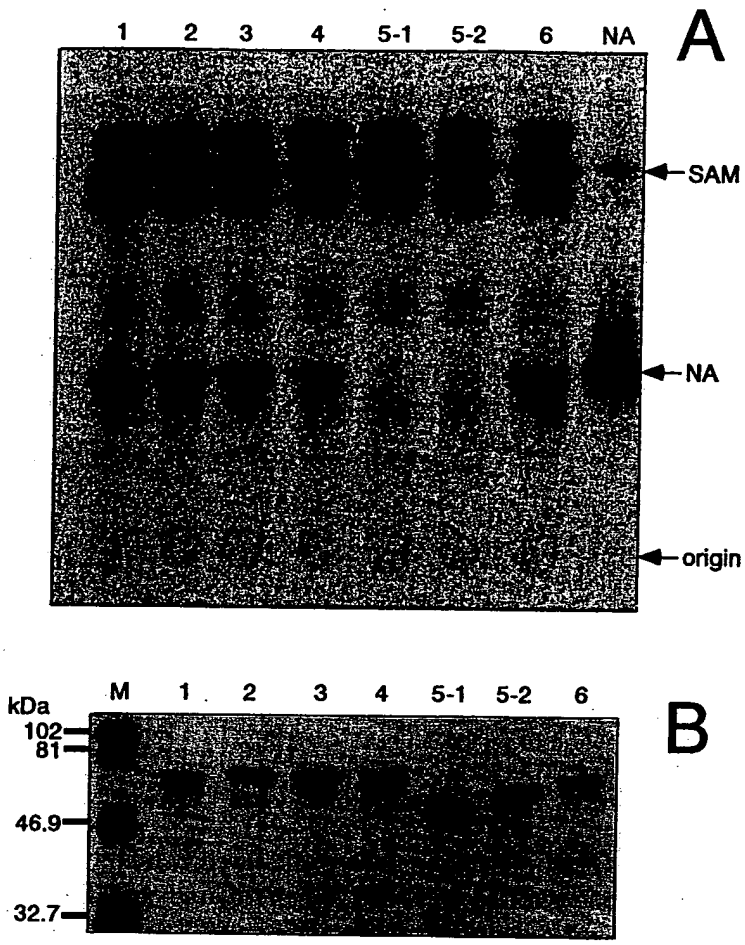

Fig. 3. Confirmation of the NAS activity of seven barley *nas* gene products. A: TLC analysis of NAS activity assay mixtures of MBP-NAS fusion proteins. 0.5 μg of each fusion protein was used for the enzyme assay. B: SDS-PAGE of MBP-NAS fusion proteins. 125 g L^{-1} acrylamide slab gels were used for SDS-PAGE and 0.5 μg of each sample was loaded. The gels were stained with CBB. Lane 1, HvNAS1; lane 2, HvNAS2; lane 3, HvNAS3; lane 4, HvNAS4; lane 5-1, HvNAS5-1; lane 5-2, HvNAS5-2; lane 6, HvNAS6; NA, standard nicotianamine; M, molecular weight marker.

SDS-PAGE (Fig. 3B). The molecular weight of MBP was about 40 kDa, and the deduced molecular weights of HvNAS1, 2, 3, 4, 5-1, 5-2, and 6 were 35.1, 35.8, 36.0, 35.4, 28.8, 30.1, and 35.4 kDa, respectively (Higuchi et al. 1999). Except for MBP-HvNAS3, the molecular weight of the major band in each lane coincided with the deduced molecular weight. The minor band just above the major band in lane 3 coincided with the deduced molecular weight of MBP-HvNAS3. In each lane, proteins with molecular weights lower than the deduced values were assumed to be degradation products, because anti-NAS antibodies recognized these proteins (data not shown).

Although MBP was stable in *E. coli*, MBP-NAS was vulnerable to degradation in *E. coli*. Moreover, the amount of MBP-NAS produced in *E. coli* was lower than that of MBP alone. Frequently, the recombinant *E. coli* strains stopped producing MBP-NAS. Therefore, NAS was considered to be toxic to *E. coli*. Since S-adenosylmethionine (SAM) is a substrate of NAS, overexpression of NAS could reduce the size of the SAM pool, which might inhibit the growth of *E. coli*. Interestingly, MBP-HvNAS5, which lacked the NAS activity, was produced rather stably. NAS was also vulnerable to degradation by proteases during extraction from barley roots (Higuchi et al. 1999). The NAS activity may be strictly regulated to prevent the deficiency of SAM, which is an essential precursor for other metabolites, and the excess NAS in Fe-deficient barley roots may be degraded immediately.

Identification of six *nas* gene products on the 2D-PAGE gel

NAS protein should be cleaved from MBP-NAS by Factor Xa according to the manufacturer's manual for the pMAL kit. However, since Factor Xa could not cleave NASs from MBP-NASs, NASs without any tag were expressed using pET-16b. Recombinant NASs were purified by SDS-PAGE and electroelution and then applied to 2D-PAGE. The N-terminal amino acid sequence of each recombinant NAS recovered from SDS-PAGE was sequenced with a protein sequencer. HvNAS5 was used as an internal standard to identify other protein spots, because its molecular weight is quite different from that of other NASs and it can be easily distinguished from others on 2D-PAGE. HvNAS1, 2, 3, 4, and 6 were

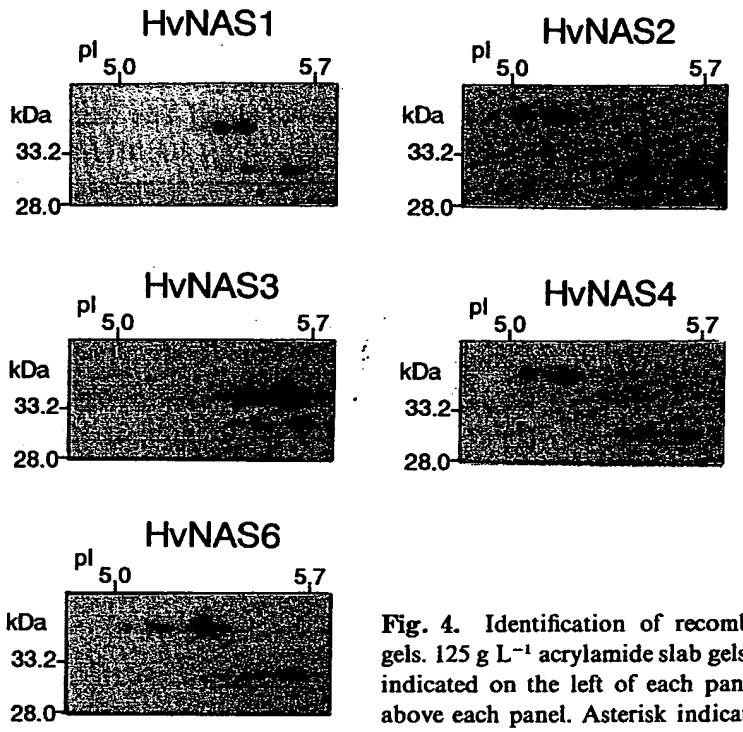

Fig. 4. Identification of recombinant NAS proteins on 2D-PAGE gels. 125 g L^{-1} acrylamide slab gels were used. The molecular weights are indicated on the left of each panel, while the pI values are indicated above each panel. Asterisk indicates HvNAS5.

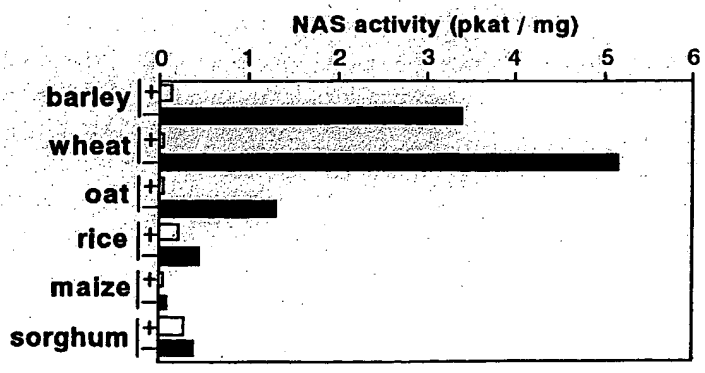

Fig. 5. NAS activity. Root materials were derived from 12 plants in each species. 0.5 g fresh weight was extracted from frozen well-homogenized roots. Each value is a mean ($n=2$). Open pattern: control plant. Closed pattern: Fe-deficient plant.

individually applied to 2D-PAGE with HvNAS5, then the gels were stained with CBB (Fig. 4). The molecular weights and pI values of each NAS closely matched the deduced values

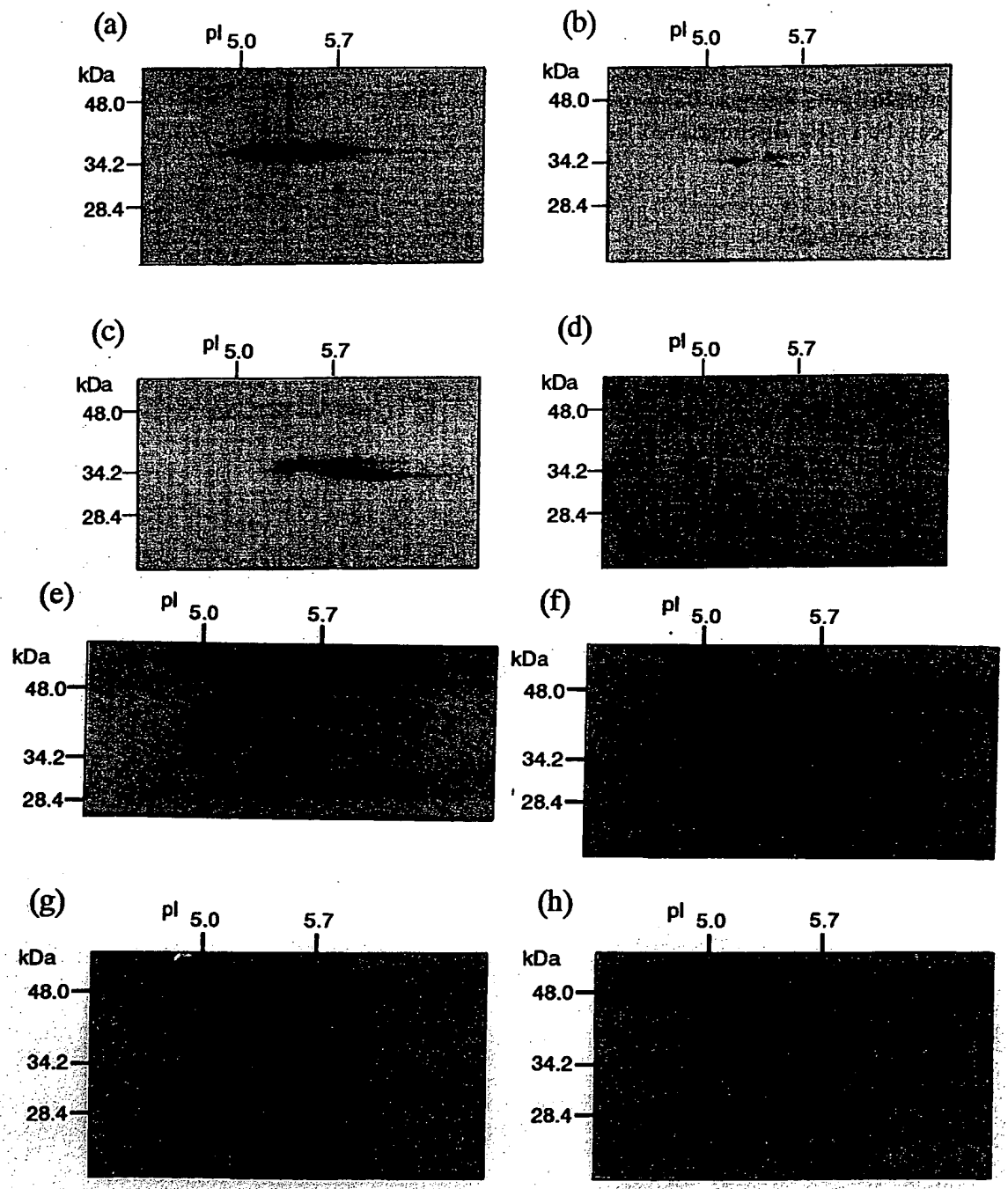

Fig. 6. Western blot analysis of total protein extracted from several graminaceous plants with TCA/acetone. 125 g L^{-1} acrylamide slab gels were used for 2D-PAGE and 200 μg of each root protein was loaded. (a) and (b), barley; (c) and (d), wheat; (e) and (f), oats; (g) and (h), rice; (i) and (j), maize; (k) and (l), sorghum; (a), (c), (e), (g), (i), and (k), Fe-deficient roots; (b), (d), (f), (h), (j), and (l), control roots. The molecular weights are indicated on the left of each panel, while the pI values are indicated above each panel.

(Higuchi et al. 1999). However, the molecular weight of recombinant HvNAS3 was lower than the deduced value. Therefore, the position of HvNAS3 on the 2D-PAGE gel could not be determined exactly. The smaller molecular weight of recombinant HvNAS3 coincided with the results shown in Fig. 3B. The C-terminal region of HvNAS3 may be vulnerable to proteases in *E. coli*.

Each of the six recombinant NAS were dispersed into three spots on the 2D-PAGE gels. Each set of 3 spots showed the same molecular weight, but different pI values. The N-terminal amino acid sequence of each recombinant NAS recovered from SDS-PAGE was sequenced with a protein sequencer, and the sequences for each set of 3 spots were identical. Acid phosphatase or alkaline phosphatase treatments had no effect. Presently, it is not clear why each recombinant NAS was separated into 3 spots on the 2D-PAGE.

NAS in various cereals

NAS activity was detected in the roots of both Fe-deficiency tolerant (barley, wheat, and oats) and Fe-deficiency susceptible (rice, maize, and sorghum) cereals (Römheld 1987) (Fig. 5). In all the species, Fe-deficiency induced NAS activity. The NAS activity ranged from high in barley, wheat, and oats, to low in rice, maize, and sorghum. Since the use of E-64 as a protease inhibitor improved the procedure applied to extract NAS proteins (Higuchi et al. 1999) and the activity was detected in the crude extracts directly, the difference in the NAS activity of each species was more distinct than that previously reported (Higuchi et al. 1996a).

Western blot analysis of each graminaceous plant using anti-NAS polyclonal antibody is shown in Fig. 6. NAS-like proteins were detected in all the species tested. The amount of each NAS homologue was determined from the size of the spots. The number or amount of each NAS homologue was correlated with the differences in NAS activity; in the subfamily

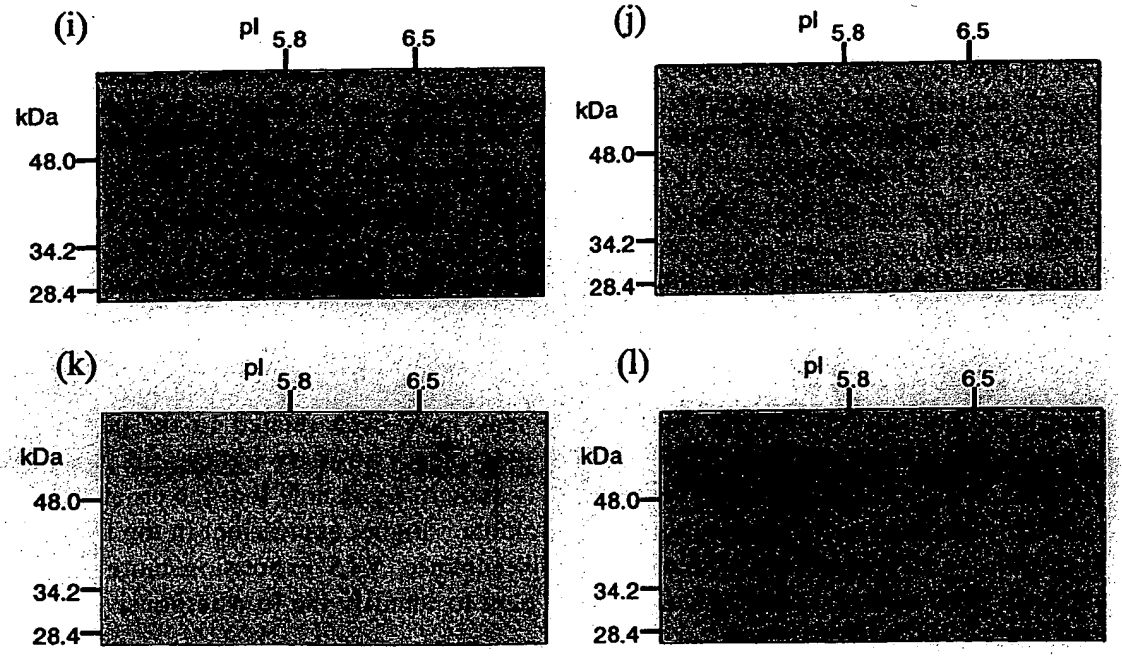

Fig. 6. Continued.

Pooideae (barley, wheat, and oats), Fe-deficiency strongly induced several isoforms of NAS or NAS-like proteins, and the isoform patterns were similar. On the other hand, in rice and the subfamily Panicoideae (maize and sorghum), Fe-deficiency weakly induced NAS or NAS-like proteins, and there were a few detectable spots. In the subfamily Pooideae, the molecular weights of NAS were about 35 kDa. However, in addition to about 35 kDa proteins, a NAS-like protein with a molecular weight of approximately 40 kDa was observed in control rice and NAS-like proteins with a molecular weight of approximately 70 kDa were observed in maize and sorghum. Moreover, the pI values of the NAS-like proteins in maize and sorghum were higher than those in the subfamily Pooideae.

Rice had fewer isoforms than the Pooideae subfamily, and there was a small amount of NAS-like protein. We identified NAS-like rice EST clones in the database, and their sequences were very similar to barley NAS. Therefore, the low NAS activity in rice may simply result from a lower level of NAS gene expression. The pI values of NAS-like proteins in the subfamily Panicoideae were different from those in the subfamily Pooideae. It is possible that the amino acid sequences of these proteins are not so similar to those of barley NAS, and then the relative activity of NAS in the subfamily Panicoideae is lower than that in the subfamily Pooideae.

Arabidopsis thaliana is a species that uses the strategy-I mechanism of iron acquisition. NA is also found in strategy-I plants, where it is proposed to play a key role as an endogenous chelator of divalent metal cations (Stephan et al. 1994). However, Fe-deficiency does not enhance NAS activity in strategy-I plants such as tobacco (Higuchi et al. 1995) and tomato (Higuchi et al. 1996b). In rice, maize, and sorghum, the amounts of NAS protein and activity were not so low in control roots, and were weakly induced by Fe-deficiency.

We also found three *nas* gene homologues in the *A. thaliana* genome database (accession no., AB005245, AC003114, AB011476). The coding regions of two of the NAS homologues, AB005245 and AB011476, were very similar to each other and both were located on chromosome 5. Predicted pI values were 6.28 and 6.14. On the other hand, the AC003114 sequence was different, and was located on chromosome 1. The predicted pI value was 5.66. Three isoforms of NAS were tentatively identified in rice, maize, and sorghum (Fig. 6). We found 19 NAS-like rice EST clones in the database, which were classified into two types with very similar putative coding regions. The program Clustal W categorized barley NASs into three types: Type 1, *nas1*; Type 2, *nas2, 3, 4*, and *6*; Type 3, *nas5*. Therefore, it is assumed that originally two types of *nas* genes may have been present in higher plants and that the copy number of one of these tend to increase. The origin of *nas5* in barley remains to be elucidated.

Based on the data described above, it is possible that the ancestor of the Gramineae acquired the ability to induce the expression of *NAS* in the case of Fe-deficiency, and subsequently both the copy number and the level of expression of *NAS* increased in the process of evolution of the subfamily Pooideae. Comparison of rice, Pooideae, and Panicoideae genomes showed that the rice genome may have been related to the genome of ancestral grass (Devos and Gale 1997). Since Fe-deficiency strongly induces all NAS-like proteins in the Pooideae, as shown in Fig. 6, it is difficult to assume that the cis-element of each *NAS* had been independently altered to promote a higher expression. In the Pooideae, iron-responsive trans-elements of *NAS* probably enhance *NAS* expression more strongly than in the Oryzodae and the Panicoideae. We plan to identify the iron responsive cis and trans elements of *NAS* in future.

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Nicotianamine synthase gene expression differs in barley and rice under Fe-deficient conditions

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Summary

Nicotianamine (NA) is an intermediate in the biosynthetic pathway of the mugineic acid family phytosiderophores (MAs), which are crucial components of the iron acquisition apparatus of graminaceous plants. In non-graminaceous plants, NA is thought to be an essential chelator for metal cation homeostasis. Thus NA plays a key role in Fe metabolism and homeostasis in all higher plants. Nicotianamine synthase (NAS, EC 2.5.1.43) catalyzes the trimerization of S-adenosylmethionine to form one molecule of NA. Barley, a plant that is resistant to Fe deficiency, secretes large amounts of MAs, whereas rice, a plant that is susceptible to Fe deficiency, secretes only small amounts. In this study we isolated a genomic fragment containing *HvNAS1* from barley and three rice cDNA clones, *osnas1*, *osnas2* and *osnas3*, from Fe-deficient rice roots. We also isolated a genomic fragment containing both *OsNAS1* and *OsNAS2*. In contrast to barley, in which Fe deficiency induces the expression of *NAS* genes only in roots, Fe deficiency in rice induced *NAS* gene expression in both roots and chlorotic leaves. The amounts of endogenous NA in both the roots and leaves were higher than in barley. We introduced barley genomic DNA fragments containing *HvNAS1* with either 9 or 2 kb of the 5′-flanking region into rice, using *Agrobacterium*-mediated transformation. Fe deficiency induced *HvNAS1* expression in both roots and leaves of the transgenic rice, as occurs with rice *NAS* genes. Barley and rice *NAS* genes are compared in a discussion of alteration of the *NAS* genes during adaptation to Fe deficiency.

Keywords: Fe deficiency, heterologous expression, mugineic acid-family phytosiderophores, nicotianamine, nicotianamine synthase gene, transgenic rice.

Introduction

Graminaceous plants secrete iron-chelating mugineic acid-family phytosiderophores (MAs) from their roots to solubilize iron in the rhizosphere (Takagi, 1976). The amount of MAs secreted increases dramatically under Fe deficiency stress. In graminaceous plants, tolerance to Fe deficiency is thought to depend on the amount of MAs secreted from their roots (Marschner *et al.*, 1987; Römheld, 1987; Singh *et al.*, 1993; Takagi *et al.*, 1984). For example, rice secretes small amounts of MAs and is susceptible to Fe deficiency, whereas barley secretes relatively high levels of MAs and can tolerate Fe deficiency. Nicotianamine (NA) is a key intermediate in the biosynthesis of MAs (Shojima *et al.*, 1989; Shojima *et al.*, 1990), and chelates various metal cations including Fe^{2+} and Fe^{3+} (von Wiren *et al.*, 1999). In dicots, which do not secrete MAs, NA is thought to play a role in metal metabolism (Becker *et al.*, 1998; Pich and Scholz, 1996). Thus NA is a key substance in Fe metabolism in all higher plants.

Increases in the activities of both nicotianamine synthase (NAS, EC 2.5.1.43) and nicotianamine aminotransferase (NAAT) are crucial for the enhanced secretion of MAs by Fe-deficient graminaceous plants, and therefore for Fe deficiency tolerance (Higuchi *et al.*, 1996a; Kanazawa *et al.*, 1994). Recently, we purified NAS and NAS-like proteins

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from Fe-deficient barley roots, and isolated seven related cDNA clones encoding NAS in barley (Higuchi *et al.*, 1999a; *hvnas7*, accession number AB019525). Western blot analysis showed that there were several NAS-like proteins in barley, wheat, oats, rice, maize and sorghum (Higuchi *et al.*, 1999b). Furthermore, database searches revealed that almost all higher plants have *NAS* homologues. Functional *NAS* genes were isolated from tomato (Ling *et al.*, 1999) and *Arabidopsis* (Suzuki *et al.*, 1999), in addition to another two barley cDNAs (Herbik *et al.*, 1999). In contrast to graminaceous plants, NAS activity is not induced by Fe deficiency in dicots (Higuchi *et al.*, 1995; Higuchi *et al.*, 1996b). Among graminaceous plants, the level of NAS activity is highly correlated with that of secreted MAs (Higuchi *et al.*, 1996a). Thus, while NA is a key substance in Fe metabolism in all higher plants, the expression patterns of the *NAS* genes may differ depending on the plant species. The details and regulatory mechanisms of *NAS* gene expression need to be fully elucidated in order for Fe metabolism to be fully understood.

To compare the properties of *NAS* genes from Fe deficiency-tolerant barley and Fe deficiency-susceptible rice, we isolated a barley genomic DNA fragment containing *HvNAS1*, three rice *NAS* cDNA clones, and a rice genomic DNA fragment containing *OsNAS1* and *OsNAS2*. The possible functions of rice NA and internal MAs, and alterations in the *NAS* gene family during adaptation to Fe deficiency, are discussed.

Results

Cloning rice NAS cDNAs

The central 555 bp region in barley *hvnas1* (Higuchi *et al.*, 1999a) was used as a probe to screen the cDNA library, and 20 positive clones were obtained. Sequence analysis of these clones identified the presence of three distinct cDNA clones, designated *osnas1*, *osnas2* and *osnas3*.

The *osnas1* open reading frame encoded a 332 amino acid polypeptide with a predicted molecular weight of 34.9 kDa and a predicted pI value of 4.96. These values corresponded well with those for one of two major rice NAS-like polypeptides detected by Western blot analysis (Higuchi *et al.*, 1999b). The deduced amino acid sequences of two rice EST clones (accession numbers D23792and D24790) matched portions of OsNAS1. The *osnas2* open reading frame encoded a 325 amino acid polypeptide with a predicted molecular weight of 34.4 kDa and a predicted pI value of 5.62. These values matched the second rice NAS-like polypeptide detected on the Western blot mentioned above. The sequence of OsNAS2 was similar to that of OsNAS1, except for the C-terminal region. The *osnas3* open reading frame encoded a 343 amino acid polypeptide with a predicted molecular weight of 37.1 kDa and a predicted pI value of 5.48. The deduced amino acid sequence of *osnas3* was slightly different from those of *osnas1* and *osnas2*, and its N-terminal region was longer. The amino acid sequence similarity of these NAS genes with other NAS is indicated in the phylogenic tree (Figure 1).

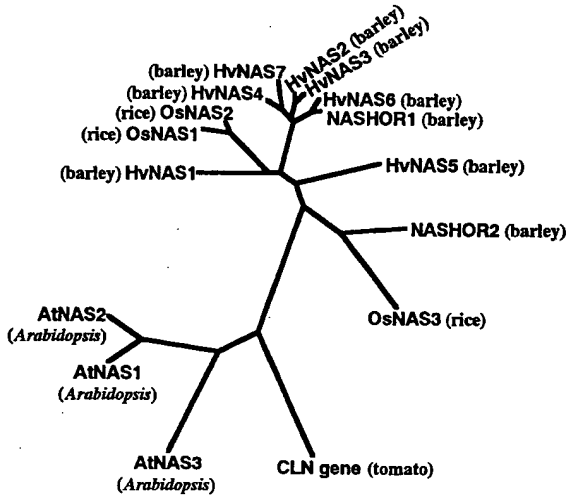

Figure 1. The unrooted phylogenic tree for the NAS amino acid sequences of which ORFs are confirmed.
The calculation was performed using the program CLUSTAL W based on the neighbor-joining method and visualized with the program TREEVIEW. Accession numbers are: AB010086(HvNAS1), AB011265(HvNAS2), AB011264(HvNAS3), AB011266(HvNAS4), AB011268(HvNAS5), AB011269(HvNAS6), AB019525(HvNAS7), AB021746(OsNAS1), AB023818(OsNAS2), AB023819(OsNAS3), AB021934(AtNAS1), AB021935(AtNAS2), AB021936(AtNAS3), AJ242045(CLN), AF136941(NASHOR1), and AF136942(NASHOR2).

To confirm the enzymatic activity of the *osnas1* gene product, it was expressed as a maltose-binding protein (MBP) fusion in *Escherichia coli*. The bacterial strain containing the OsNAS1 expression vector was induced with isopropyl-beta-D-thiogalactopyranoside (IPTG), and the fusion protein was purified by affinity chromatography and analyzed for NAS activity by TLC. The activity of purified MBP-HvNAS1 (Higuchi *et al.*, 1999a) was also analyzed at the same time. The relative activities of MBP-OsNAS1 and MBP-HvNAS1 were equal (data not shown).

Northern blot analysis of NAS *expression in barley and rice*

To compare the transcript levels of *NAS* genes in barley (*Hordeum vulgare* L. cv. Ehimehadaka no. 1) and rice (*Oryza sativa* L. cv. Nihonbare), Northern analysis was performed. The probes used for Northern analysis were the full-length *hvnas1* and *osnas1* ORFs. The *hvnas* sequences are very similar to each other; thus almost all the *hvnas* transcripts in barley were detected using the *hvnas1* ORF. Similarly, almost all the *osnas* transcripts in rice were also detected using the *osnas1* ORF. We also

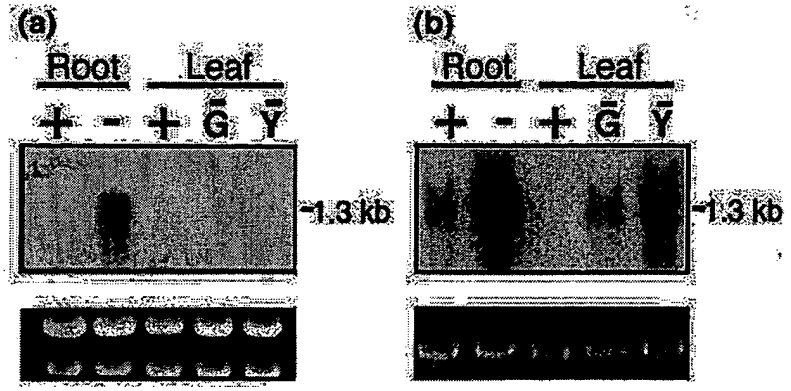

Figure 2. Northern blot analysis of barley total RNA using the *hvnas1* ORF (a) and of rice total RNA using the *osnas1* ORF (b) as probes.
RNA was extracted from the roots or leaves of Fe-deficient plants after 2 weeks' Fe-deficiency treatment or from control plants. +, Control plants; –, Fe-deficient plants; G, green leaves of Fe-deficient plants; Y, yellow leaves of Fe-deficient plants. Each lane was loaded with 20 μg total RNA. The lower panel indicates rRNA.

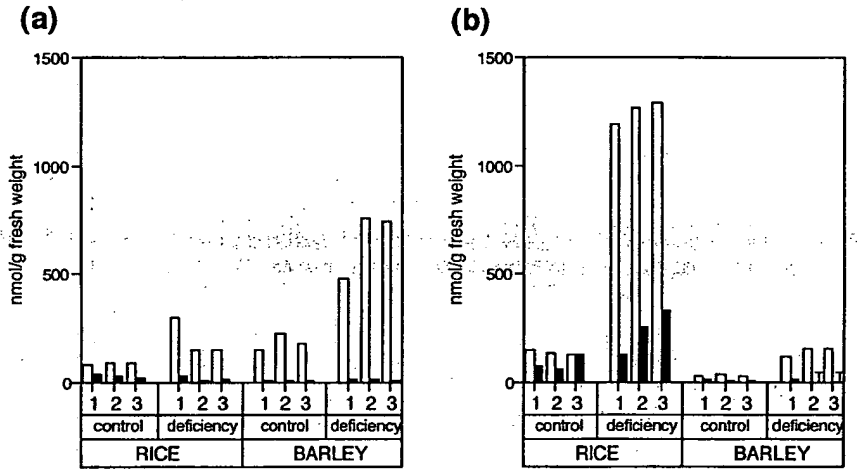

Figure 3. Endogenous NA and MAs in barley and rice.
(a) Roots; (b) leaves. Values from three independent experiments are shown. MAs include DMA, MA and epi-HMA. T, trace; white bars, MAs; black bars, NA.

used a short fragment of the highly conserved region as a probe for both barley and rice, but this fragment gave weak signals. The results using the full-length probes are shown in Figure 2.

Deficiency in Fe induced *osnas* expression in both roots and leaves, especially chlorotic leaves (Figure 2b). A weak signal was detected in control rice roots (Figure 2b), while no signal was detected in control barley roots (Figure 2a). Due to the very strong signal in Fe-deficient barley roots, the exposure time of Figure 2(a) was reduced. This indicated that the transcript level of *nas* was much higher in barley roots than in rice roots. Even with prolonged exposure, no signal was detected in barley leaves or control barley roots (data not shown).

Endogenous NA and MAs in barley and rice

Since the patterns of *NAS* expression in barley and rice differ, it is conceivable that the NAS activities and the amounts of endogenous NA and MAs may also differ. To determine whether this is the case, HPLC was used to analyze the amounts of NA and MAs extracted with hot water after MAs secretion. It was confirmed that barley secreted several times more MAs than rice, which concurs with results reported previously (Kanazawa *et al.*, 1994).

As expected, Fe deficiency increased the amounts of MAs in the roots of both barley and rice (Figure 3a, open pattern); the amounts in barley were two to three times higher under both Fe-sufficient and Fe-deficient conditions. Only a trace amount of NA was found in barley roots, less than is found in rice roots (Figure 3a, closed pattern). Levels in MAs in both rice and barley leaves increased under Fe deficiency (Figure 3b, open pattern); the level was several times higher in rice than in barley. Barley leaves contained only a trace amount of NA (Figure 3b, closed pattern). The amount of NA in rice leaves increased under Fe-deficient conditions. Overall, in comparison to barley roots, the amount of NA in rice roots was high and the amount of MAs was low.

Structures of HvNAS1, OsNAS1 *and* OsNAS2

To investigate the regulatory mechanisms of *NAS* gene expression, genomic clones were isolated from barley and

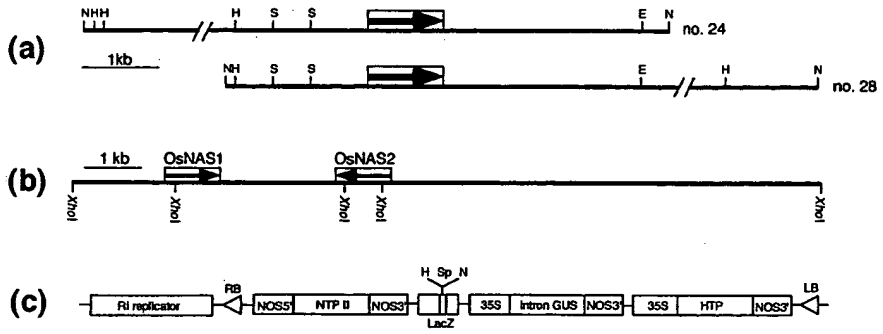

Figure 4. Structures of *HvNAS1*, *OsNAS1–2*, and vector pBIGRZ1.
(a) Two barley *HvNAS1* genomic clones. (b) Rice *OsNAS1–2* genomic clone. (c) Vector pBIGRZ1. Arrows indicate ORF. N, *Not*I; H, *Hin*dIII; S, *Sac*I; E, *Eco*RI; Sp, *Spe*I. RB, right border of T-DNA; LB, left border of T-DNA; NPTII, neomycin phosphotransferase type II gene; GUS, β-glucuronidase gene; NOS5', nopaline synthase promoter; NOS3', nopaline synthase terminator; HTP, hygromycin phosphotransferase gene; LacZ, β-galactosidase gene.

rice. A barley genomic library was screened with an *hvnas1* cDNA clone. Positive clones 24 and 28 containing *HvNAS1* (Figure 4a) were analyzed and used for transforming rice. *HvNAS1* did not have any introns. The 5′ upstream regions of clones 24 and 28 were 9 and 2 kb long, respectively; the 3′ downstream regions were 3 and 11 kb long, respectively.

A rice genomic library was screened with an *osnas1* cDNA clone. One positive clone carried both *OsNAS1* and *OsNAS2* (Figure 4b), and neither gene had introns. The two genes had the opposite orientations, with their 3′ ends separated by 2 kb. *OsNAS1* had a 1.6 kb 5′ upstream region; that of *OsNAS2* was 7.7 kb long.

Database searches revealed that known 5′ *cis*-acting regulatory elements were located upstream of *HvNAS1*, *OsNAS1* or *OsNAS2*. These included the mammalian metal-responsive element MRE1 (Stuart *et al.*, 1985); the root motif found in the promoters of *rolD* and root-specific wheat peroxidase (Elmayan and Tepfer, 1995); the MYC-binding site found in the promoters of a dehydration-responsive gene (Abe *et al.*, 1997) and ABA-inducible genes; and the core nucleotides of the low temperature (Baker *et al.*, 1994) and elicitor (Rushton *et al.*, 1996) response elements. However, no known metal nutrition-related elements were found in the promoter regions of *HvNAS1*, *OsNAS1* or *OsNAS2*.

Transgenic HvNAS1 *rice*

We constructed two binary vectors based on pBIGRZ1 for rice transformation. pBIGRZ1 is a vector that is capable of holding a large insert (Akiyama *et al.*, 1997; Figure 4c). It is useful because the transgene copy number is usually less than three, and a long intact transgene can be stably integrated into the host genome. The inserts were excised from phage clones 24 and 28 with *Not*I and cloned into the *Not*I site of pBIGRZ; the vectors were designated 9k-HvNAS1 for clone 24, and 2k-HvNAS1 for clone 28.

9k-HvNAS1 and 2k-HvNAS1 were introduced into rice by *Agrobacterium*-mediated transformation. Seven lines of vector control pBIGRZ rice, six lines of 9k-HvNAS1 rice, and eight lines of 2k-HvNAS1 rice were obtained. T_1 plants were used for further analysis. Southern analysis showed that the transgenic plants had from one to three copies of the introduced genes (data not shown).

HvNAS1 *and* OsNAS1 *expression in transgenic rice*

Northern analysis of transgenic rice using an *osnas1*-specific probe confirmed induction of *OsNAS1* in both leaves and roots (Figure 5, upper panel). Northern analysis using an *hvnas1*-specific probe showed that Fe deficiency induced expression of *HvNAS1* in both leaves and roots of transgenic rice (Figure 5, middle panel). This result indicated that the 2 and 9 kb 5′ upstream regions of the *HvNAS1* transgenes had the ability to regulate *HvNAS1* expression in both tissues in response to Fe deficiency.

With the *hvnas1*-specific probe, two bands, with similar molecular weights, were detected only in Fe-deficient leaves of both 9k-HvNAS1 and 2k-HvNAS1 rice. The molecular weight of one of the two bands was less than that of *hvnas1*. Since the specific probe designed from the 3′ untranslated region detected the bands, there is no possibility that degradation from the 3′ end of the mRNA caused by some signal sequences gave incomplete *hvnas1* mRNA.

NAS proteins in transgenic rice roots

Anti-NAS polyclonal antibodies that recognize almost all graminaceous NAS proteins were used for detection, after separating polypeptides by two-dimensional SDS–PAGE

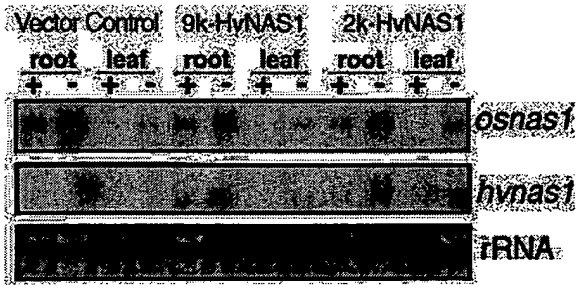

Figure 5. Northern blot analysis of transgenic rice.
hvnas1-specific and *osnas1*-specific probes were used. Total RNA was extracted from the roots or leaves of Fe-deficient plants after 2 weeks' Fe-deficiency treatment or from control plants. +, control plants; −, Fe-deficient plants. Each lane was loaded with 20 μg total RNA.

(Higuchi *et al.*, 1999b). HvNAS1, OsNAS1, and OsNAS2 were all clearly distinguishable (Figure 6). Since the Rubisco content of total leaf protein is very high, the relatively small amounts of NAS proteins in the leaves could not be detected. Based on the intensities of three spots that accumulated under conditions of Fe deficiency, the amount of HvNAS1 protein in transgenic rice roots was estimated to be almost the same as that of OsNAS1 and OsNAS2.

Discussion

Contribution of NAS to Fe deficiency tolerance

Under Fe deficiency, the expression level of *nas* was higher in barley roots than in rice roots (Figure 2). One molecule of MAs is synthesized from one molecule of NA, thus the total of NA and MAs gives the total amount of NA synthesized. Figure 3(a) shows that NA synthesis activity was much higher in barley root than in rice root. These results coincide with the knowledge that barley has high NAS activity and secretes much more MAs.

To clarify why rice has low total NAS activity, the activity of purified MBP-HvNAS1 (Higuchi *et al.*, 1999a) was compared with that of MBP-OsNAS1. Previously, we reported that the relative activities of MBP-HvNAS1, MBP-HvNAS2, MBP-HvNAS3, MBP-HvNAS4 and MBP-HvNAS6 were equal, and all of these NAS proteins were induced by Fe deficiency in barley roots (Higuchi *et al.*, 1999b). Therefore we chose HvNAS1 as a representative of barley NAS. On the other hand, we chose MBP-OsNAS1 as the main NAS in rice for the following reason. Ten copies of *osnas1* were cloned from the cDNA library, but only four copies of *osnas2* and one of *osnas3* were cloned. Repeated Western blot analysis demonstrated that both OsNAS1 and OsNAS2 were induced by Fe deficiency all the time. However, the level of OsNAS1 induction was always high, while that of OsNAS2 fluctuated (data not shown). The relative activities of MBP-OsNAS1 and MBP-HvNAS1 were equal (data not shown). Thus the low total NAS activity seen in rice may be the consequence of the small amount of enzyme present.

It was thought that MAs biosynthesis occurs only in roots, as roots secrete the MAs. Previously, NAS activity was not detected in barley leaves (Higuchi *et al.*, 1994), and *HvNAS* expression in response to Fe deficiency was induced only in roots (Higuchi *et al.*, 1999a; Figure 2a in this paper). Although the concentration of MAs in rice leaves is rather high (Mori *et al.*, 1991), these MAs are believed to be translocated from the roots in a complex form with Fe. In this study we demonstrated that rice induced NAS under Fe deficiency, not only in roots but also in leaves (Figure 2). The induction was greater in chlorotic leaves than in green leaves. Moreover, in barley *NAS* expression was strictly repressed under Fe-sufficient conditions, while in rice *NAS* genes were constitutively expressed at relatively high levels. These results suggest that rice NAS may have roles other than just the biosynthesis of MAs. Three molecules of high-energy S-adenosylmethionine (SAM) are consumed to form one molecule of NA, indicating that NA may have a critical role in rice under both Fe-sufficient and Fe-deficient conditions.

In strategy I dicotyledonous plants, NAS activity is not induced by Fe deficiency (Higuchi *et al.*, 1995; Higuchi *et al.*, 1996b). Based on analysis of the tomato mutant *Chloronerva*, NA is known to be essential for Fe transport from vein to intervein (Scholz, 1965). An *in vitro* experiment suggests that NA chelates Fe^{3+} as well as divalent metal ions in plants (von Wiren *et al.*, 1999). NA itself may regulate Fe transport in rice, as it does in strategy I plants. Otherwise, MAs in rice leaves may contribute to solubilizing and recycling deposited Fe. However, the possibility remains that the adaptation of rice to Fe deficiency is insufficient, and that it has not yet evolved root-specific MAs synthesis.

While *NAS* expression was not observed in Fe-sufficient barley roots (Figure 2), MAs were detected (Figure 3). It is thought that barley might also take up Fe with MAs under Fe-sufficient conditions. Very low *NAS* expression might occur, and MAs might accumulate in the roots under Fe-sufficient conditions. The amount of NA in rice roots decreased in response to Fe deficiency (Figure 3), and this may be caused by the conversion of NA to 2′-deoxymugineic acid (DMA) and subsequent DMA secretion. The MAs in rice leaves may be synthesized in the leaves, and may not contribute to secretion from the roots. Overall, in comparison with barley roots, the amount of NA in rice roots was high and the amount of MAs was low (Figure 3). It is possible that in barley all NA is converted to MAs, whereas in rice some endogenous NA remains. This suggests that the supply of SAM substrate is sufficient in both barley and rice, but in rice the capacity for DMA

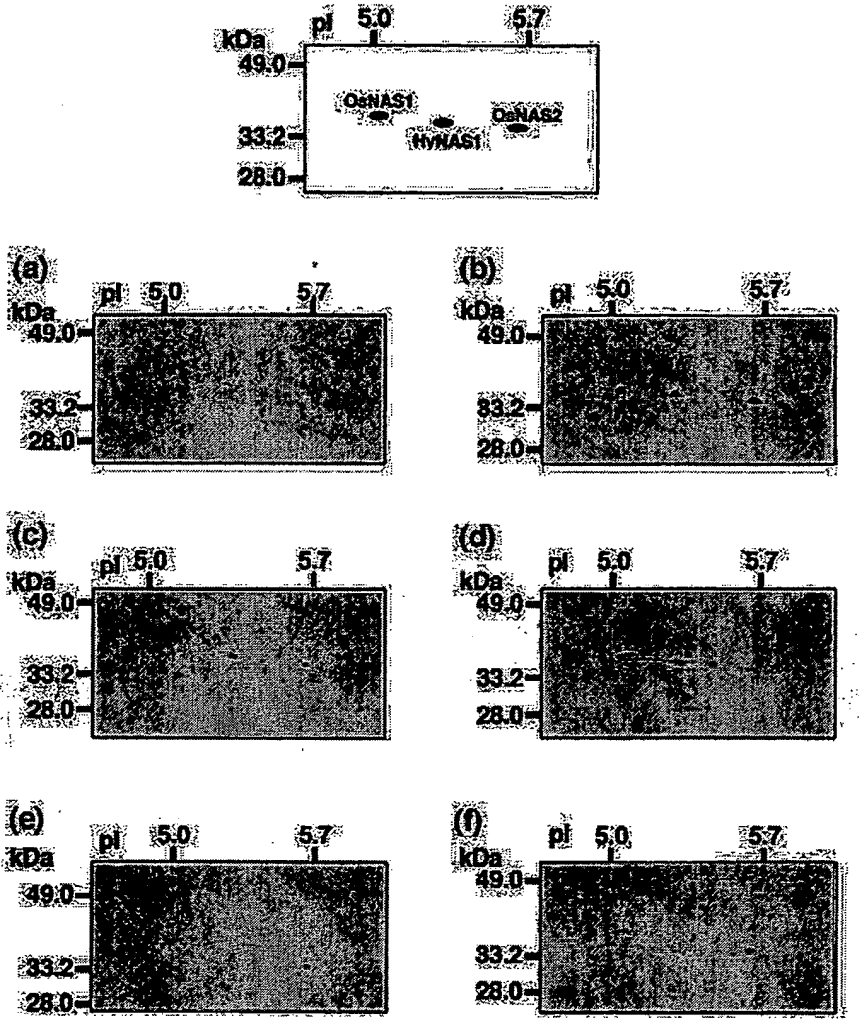

Figure 6. Western blot analysis of transgenic rice.
Total protein extracted from roots of Fe-deficient plants after 2 weeks' Fe-deficiency treatment or from control plants with TCA/acetone. 125 g l^{-1} acrylamide slab gels were used for 2D-PAGE and 200 μg of each root protein was loaded. (a,b) 9k-HvNAS1; (c,d) 2k-HvNAS1; (e,f) vector control. (a,c,e) Control roots; (b,d,f) Fe-deficient roots. Molecular weights are indicated on the left of each panel, pI values above each panel.

synthesis from NA is lower than that for NA synthesis. Thus rice secretes fewer MAs than barley.

Alteration of NAS *gene expression during adaptation to Fe deficiency*

Roots secrete large amounts of MAs; therefore strong and specific induction of *NAS* genes in the roots is favorable in order to secrete large amounts of MAs in response to Fe deficiency. As shown in Figure 3, large amounts of MAs are synthesized in rice leaves, but this synthesis may not contribute to the secretion of MAs from roots. Barley may use concentrated root *NAS* expression to efficiently secrete large amounts of MAs from that tissue.

The expression pattern of *HvNAS1* in transgenic rice (Figure 5) strongly suggests that organ-specific induction of *NAS* in barley and rice is due to the actions of different *trans*-acting regulatory elements, and that the *HvNAS1* and *OsNAS1* promoters function equally well in rice. In barley, we speculate that *trans*-acting regulatory elements that induce *NAS* gene expression in response to Fe deficiency are present only in the roots, or that some suppressor mechanism negates their effects in the leaves, because it would be difficult to modulate gene expression by simultaneously co-ordinating all of the 5′ *cis*-acting elements found upstream from the barley *NAS* genes. Western analysis (Figure 6) suggests also that the promoter activities of *HvNAS1*, *OsNAS1* and *OsNAS2* in rice are almost equal and function equally well in rice. Unknown *cis*-acting elements conserved in both barley and rice may participate in *NAS* regulation. However, the presence of incomplete *hvnas1* mRNA in Fe-deficient rice leaf (Figure 5) suggests that the difference in *cis*-elements between *hvnas1* and *osnas1* also affects organ-specific expression.

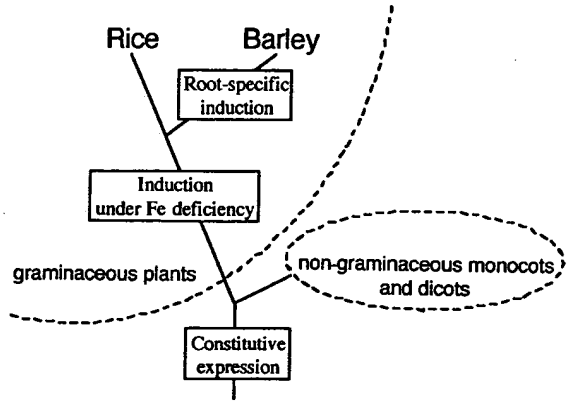

Figure 7. Proposed process of alteration in the NAS gene family adapted to Fe deficiency.

Rice *OsNAS1* and *OsNAS2* are highly homologous, as are *Arabidopsis AtNAS1* and *AtNAS2* (Figure 1). The barley genes *HvNAS2, 3, 4, 6 and 7*, and *NASHOR1*, also share high homology (Figure 1), suggesting that amplification of these genes occurred quite recently. A search of the *Arabidopsis* database revealed that *AtNAS1* and *AtNAS2* (accession numbers AB005245and AB011476) are both located on chromosome V; they are separated by ≈100 cM. *AtNAS3* (accession number AC003114) is located on chromosome I, and a short *NAS*-like sequence (accession number AL078465) is located on chromosome IV. It is possible that *NAS* gene structure promotes unequal crossing over (Ohno, 1970) and that the *NAS* genes are tandemly located, as is the case with *OsNAS1* and *OsNAS2* (Figure 4b). We speculate that amplification of *NAS* genes has frequently occurred in plant genomes, but useless *NAS* genes are inactivated and lost from the genome. As having a high number of functional *NAS* genes would be favorable for secreting large amounts of MAs, it is possible that the Pooideae maintain many *NAS* genes. In the case of paddy rice, which evolved under Fe-sufficient conditions, excess *NAS* genes may have been eliminated.

In conclusion, it is possible that the ancestor of the Graminaceae acquired the ability to induce *NAS* expression in response to Fe deficiency, for the purpose of MA synthesis. Barley then acquired root-specific induction and high *NAS* copy number during its evolution (Figure 7).

Experimental procedures

Preparation of plant materials

Wild-type and transgenic rice seeds were germinated on wet filter paper and transferred into a nutrient solution in a greenhouse with 30°C light/25°C dark periods under natural light conditions. The composition of the nutrient solution was: 0.35 mM $(NH_4)_2SO_4$, 0.18 mM Na_2HPO_4, 0.27 mM K_2SO_4, 0.36 mM $CaCl_2$, 0.46 mM $MgSO_4$, 18 μM H_3BO_3, 4.6 μM $MnSO_4$, 1.5 μM $ZnSO_4$, 1.5 μM $CuSO_4$, 1.0 μM Na_2MoO_4, and 45 μM Fe(III)-EDTA. The pH of the culture solution was adjusted to 5.5 every day with HCl or NaOH. When the sixth leaves appeared some plants were transferred to a culture solution prepared with de-ionized water that contained all nutrients except Fe. Iron-sufficient plants were cultured continuously in the standard culture solution. Two weeks after transplanting, when severe iron chlorosis appeared on newly developing leaves, the leaves and roots were harvested, crushed in liquid N_2, and stored at –80°C until further use.

Barley (*Hordeum vulgare* L. cv. Ehimehadaka no. 1) was grown in hydroponic culture as previously described (Higuchi *et al.*, 1996a). Two weeks after Fe-deficiency treatment, when severe iron chlorosis appeared on the fourth or fifth leaves, roots and leaves were harvested, crushed in liquid N_2, and stored at –80°C until further use.

Northern blot analysis

The probes used for Northern blotting were an *Eco*RI/*Not*I restriction fragment containing the full-length *osnas1* cDNA and a *Hin*dIII/*Not*I restriction fragment containing the full-length *hvnas1* cDNA. The probes used for analysis of transgenic rice were the *hvnas1* cDNA 3′ non-coding region from nucleotides 1038–1230, and nucleotides 1084–1347 of the *osnas1* cDNA 3′ non-coding region. Procedures for total RNA isolation, probe labeling and hybridization were as described previously (Higuchi *et al.*, 1999a). Radioactivity was detected using a BAS-2000 Image Analyzer (Fuji Film, Tokyo, Japan).

Extraction and detection of endogenous NA and MAs

Frozen plant materials were homogenized with a mortar and pestle and suspended in water at about 80°C for 30 min. The supernatants were concentrated and dried by evaporation. Residues were dissolved in HPLC buffer and filtered using a 0.22 μm membrane filter. NA and DMA were detected by HPLC with *o*-phthalaldehyde (Mori and Nishizawa, 1987; Shojima *et al.*, 1989). The pH of 0.15 N Li-citrate buffer was decreased to 2.60 for better separation of MAs, and the pH of 0.2 N Li-citrate buffer was increased to 3.30 for better separation of NA.

Cloning NAS genes

Poly(A)+ RNA was extracted from Fe-deficient rice roots by the guanidine method (Chomczynski and Sacchi, 1987). cDNA was synthesized using the SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning (Gibco BRL, Rockville, MD, USA) without the kit dNTPs. [10 mM dATP, 10 mM dGTP, 10 mM dTTP, 5 mM 5-methyl-dCTP] was used as the dNTP mix for first-strand synthesis and [10 mM dATP, 10 mM dGTP, 10 mM dTTP, 26 mM dCTP] was used for second-strand synthesis. The cDNA was cloned into pSPORT1 (Gibco BRL) to construct a cDNA library, which was screened with an *Apa*LI/*Xho*I fragment from the barley *HvNAS1* cDNA clone (Higuchi *et al.*, 1999a; Accession number AB010086). The probe was labeled with [α-^{32}P]dATP using the Random Primer Labeling Kit Ver. 2 (TaKaRa, Kusatsu, Japan) and the labeled DNA was purified with a ProbeQuant G-50 Micro Column (Pharmacia, Uppsala, Sweden). The isolated cDNA clones were sequenced using a Thermo Sequenase Cycle Sequencing Kit (Shimadzu, Kyoto, Japan) and a Shimadzu DSQ-2000L DNA sequencer.

The rice (var. IR36) genomic library in EMBL3SP6/T7 (Clontech, Palo Alto, CA, USA) was screened with the rice *osnas1* cDNA, cloned as described above. The barley (var. Igri) genomic library in λFixII (Stratagene, La Jolla, CA, USA) was screened with the barley *hvnas1* cDNA clone. The isolated clones were subcloned into pBluescriptII (SK-) and sequenced as described above.

Preparation of transgenic rice

We constructed two binary vectors (Figure 3) for *Agrobacterium*-mediated plant transformation. The backbone of these vectors is pBIGRZ1 (Akiyama *et al.*, 1997). The inserts were excised from the barley genomic phage clones 24 and 28 with *Not*I. Each fragment was cloned into the *Not*I site in the multi-cloning site of pBIGRZ, and these vectors were designated 9k-HvNAS1 for clone 24 and 2k-HvNAS1 for clone 28.

Transgenic rice plants were prepared according the method developed by Hiei *et al.* (1994) and modified by Akiyama *et al.* (1997). Rice (*Oryza sativa* L.) cv. Tsukinohikari was used. Four-week-old calli developed from mature seeds were immersed in the *Agrobacterium* suspension at a density of ≈3 × 10^8 cells ml^{-1} for 30 sec. After co-cultivation, selection with hygromycin was performed using 10 mg l^{-1} hygromycin B for 1 week and 50 mg l^{-1} hygromycin B for 3 weeks. Then calli were cultured on MS medium (Murashige and Skoog, 1962) containing 30 g l^{-1} sucrose, 30 g l^{-1} sorbitol, 2 g l^{-1} casamino acid, 5 mM 2-morpholinoethanesulphonic acid, 2 mg l^{-1} α-naphthaleneacetic acid, 1 mg l^{-1} kinetin, 250 mg l^{-1} Claforan, 50 mg l^{-1} hygromycin B and 0.4% gellan gum for 3 weeks, and then on MS medium containing 30 g l^{-1} sucrose, 250 mg l^{-1} Claforan, 50 mg l^{-1} hygromycin B and 0.8% agar at 28°C until regeneration, under a 16 h light/8 h dark regime. Regenerated plantlets were transferred to soil.

Western blot analysis

Polyclonal NAS antibodies and the two-dimensional electrophoresis procedure were as described previously (Higuchi *et al.*, 1999b). 200 μg total protein was loaded.

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Accession numbers AB021746 (*osnas1*), AB023818 (*osnas 2*), AB023819 (*osnas 3*), AB046401 (*OsNAS1–2*), AB022688 (region upstream from *HvNAS1* ORF) and AB023436 (region downstream from *HvNAS1* ORF).

Ref 4

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Three rice nicotianamine synthase genes, *OsNAS1*, *OsNAS2*, and *OsNAS3* are expressed in cells involved in long-distance transport of iron and differentially regulated by iron

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Summary

Nicotianamine (NA), a chelator of metals, is ubiquitously present in higher plants. In graminaceous plants, NA is a biosynthetic precursor of phytosiderophores and is thus a crucial component for iron (Fe) acquisition. Here, we show that three rice NA synthase (*NAS*) genes, *OsNAS1*, *OsNAS2*, and *OsNAS3* are expressed in cells involved in long-distance transport of Fe and that the three genes are differentially regulated by Fe. *OsNAS1* and *OsNAS2* transcripts were detected in Fe-sufficient roots but not in leaves, and levels of both increased markedly in both roots and leaves in response to Fe deficiency. In contrast, the *OsNAS3* transcript was present in leaves but was very low in roots of Fe-sufficient plants. Further, *OsNAS3* expression was induced in roots but was suppressed in leaves in response to Fe deficiency. Promoter–GUS analysis revealed that *OsNAS1* and *OsNAS2* were expressed in Fe-sufficient roots in companion cells and pericycle cells adjacent to the protoxylem. With Fe deficiency, *OsNAS1* and *OsNAS2* expression extended to all root cells along with an increase in phytosiderophore secretion. In Fe-deficient plants, *OsNAS1* and *OsNAS2* were expressed in the vascular bundles of green leaves and in all cells of leaves showing severe chlorosis. *OsNAS3* expression was restricted to the pericycle and companion cells of the roots, and in companion cells of leaves irrespective of Fe status. These results strongly suggested that NAS and NA play an important role in long-distance transport of Fe in rice plants, in addition to their roles in phytosiderophore secretion from roots.

Keywords: companion cells, Fe deficiency, long-distance transport, motor cells, mugineic acid family phytosiderophores, nicotianamine synthase.

Introduction

Iron (Fe) is an essential element required for various cellular events such as respiration, chlorophyll biosynthesis, and photosynthetic electron transfer. Fe is also a component of the Fe–S cluster present in numerous enzymes. Although abundant in soil, Fe is present mainly as oxidized Fe(III) compounds, which are poorly soluble in neutral-to-alkaline soils. Therefore, plants growing in such soils are subject to Fe deficiency. To avoid a deficit of Fe, plants have evolved two main strategies for Fe acquisition, Strategies I and II (Römheld and Marschner, 1986). All except the graminaceous plants exhibit Strategy I, releasing protons into the soil to lower its pH, inducing the expression of Fe(III) reductase to reduce Fe(III) in the rhizosphere, and taking up Fe(II) via the Fe(II) transporter. Genes for Fe(III) reductase, *FRO2* (Robinson *et al.*, 1999) and *FRO1*(Waters *et al.*, 2002), and for the Fe(II) transporter, *IRT1* (Eide *et al.*, 1996) and *IRT2* (Vert *et al.*, 2001) have been isolated from *Arabidopsis*.

In contrast, graminaceous plants, which use Strategy II, secrete mugineic acid family phytosiderophores (MAs) from their roots to solubilize rhizospheric Fe(III) (Takagi, 1976). Graminaceous plants increase the production and

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secretion of MAs in response to Fe deficiency, and Fe(III)–MA complexes are taken up through the Fe(III)–MA transporter. NA is a precursor in MA biosynthesis (Mori and Nishizawa, 1987; Shojima *et al.*, 1989, 1990). Therefore, NAS is a critical component in Fe acquisition in graminaceous plants (Figure 1). The tolerance of these plants to low Fe availability is thought to depend on the amount of MAs secreted from roots (Marschner *et al.*, 1987; Takagi *et al.*, 1984). Introducing the barley nicotianamine aminotransferase (*NAAT*) genes into rice enhances the tolerance of transgenic rice to low Fe availability, as transgenic rice plants secrete higher amounts of MAs than do wild-type plants (Takahashi *et al.*, 2001).

The *Zea mays* gene, *YS1*, encoding an Fe(III)–MA transporter has been isolated using the maize mutant *yellow stripe 1* (*ys1*) (Curie *et al.*, 2001). Interestingly, eight putative YS1-like proteins are found in the genome of *Arabidopsis*, which neither synthesizes nor uses MAs. NA, which is structurally similar to MAs and is also a strong chelator of various transition metals (von Wirén *et al.*, 1999), is ubiquitously present in higher plants (Noma and Noguchi, 1976; Noma *et al.*, 1971) including *Arabidopsis* (Figure 1). Therefore, these YS1-like proteins are thought to mediate internal transport of metals bound to NA (Walker, 2002). The functions of NA have been studied using the tomato mutant, *chloronerva*, which is characterized by Fe-deficiency symptoms such as interveinal chlorosis, excessive root branching, and retarded growth (Stephan and Grün, 1989). Grafting of the *chloronerva* mutant to the wild type normalized the mutant phenotype (Rudolph and Scholz, 1972), indicating that a substance necessary for transporting Fe is lacking in the mutant. This substance has been purified and identified as NA (Budéšínsky *et al.*, 1980). As exogenous application of NA normalized the mutant phenotype to that of the wild type, it is thought that NA is a key component in Fe homeostasis in non-graminaceous plants. Recently, Takahashi *et al.* (2003) showed that transgenic tobacco plants constitutively expressing the barley *NAAT* gene had young leaves with interveinal chlorosis and abnormally shaped flowers. Endogenous NA was consumed as a result of NAAT overproduction in the transgenic tobacco plants and both the concentration and distribution of metals in leaves and flowers were changed in these plants. A shortage of NA caused disorders in internal metal transport, leading to these abnormal phenotypes. These results suggest that a shortage of NA also impaired the functions of metal-requiring proteins, including transcription factors.

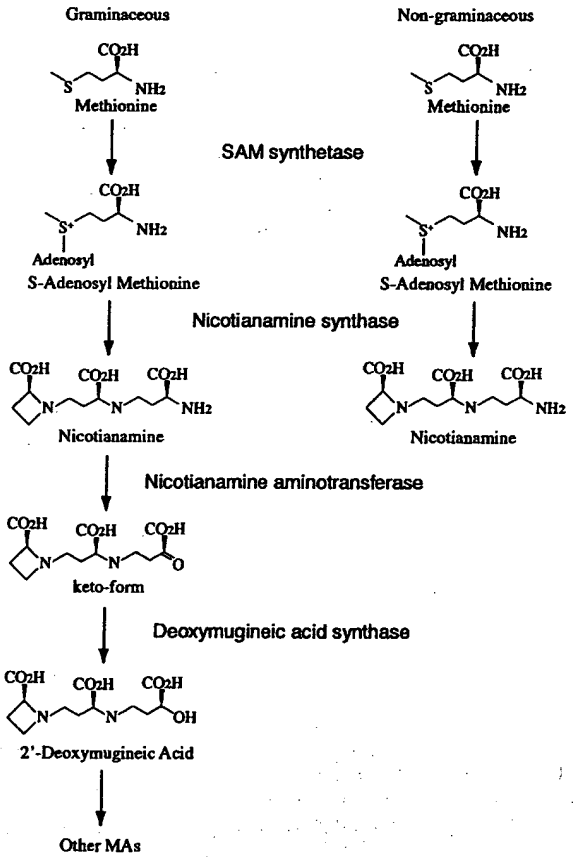


Figure 1. The biosynthetic pathway of NA and DMA.
Methionine is activated to SAM by SAM synthetase, and three molecules of SAM are combined by NAS to form NA in both graminaceous and non-graminaceous plants. In graminaceous plants, the amino group of NA is transferred by NAAT, and the resultant keto form is reduced to DMA and other MAs. Rice produces DMA.

NAS and NAS-like proteins have been purified from Fe-deficient barley roots, and seven cDNAs encoding NASs were first isolated from barley by Higuchi *et al.* (1999). Subsequently, additional *NAS* genes have been isolated from tomato (*CLN*; Ling *et al.*, 1999), *Arabidopsis* (*AtNAS1*, *AtNAS2*, and *AtNAS3*; Suzuki *et al.*, 1999), barley (*NAShor1* and *NAShor2*; Herbik *et al.*, 1999), rice (*OsNAS1*, *OsNAS2*, and *OsNAS3*; Higuchi *et al.*, 2001) and maize (*ZmNAS1*, *ZmNAS2*, and *ZmNAS3*; Mizuno *et al.*, 2003). Higuchi *et al.* (2001) showed that the expression of *OsNAS1* is induced in response to Fe deficiency in both roots and leaves, and especially in chlorotic leaves. *OsNAS1* expression was also observed in Fe-sufficient roots, but no expression was detected in Fe-sufficient leaves. Amounts of both OsNAS1 and OsNAS2 proteins were increased in Fe-deficient roots.

To elucidate the physiological functions of the three *OsNAS* genes in rice, we examined the expression of *OsNAS1*, *OsNAS2*, and *OsNAS3* by Northern blot analysis and quantitative RT-PCR, and revealed that three *NAS* genes were differentially regulated by Fe. We also examined the localization of *OsNAS1*, *OsNAS2*, and *OsNAS3* expressions through promoter–GUS analysis and found that the expression of all three *NAS* genes was found in the cells participating in long-distance transport of Fe.

Results

Fe differentially regulates three OsNAS *genes*

Northern blot analysis showed that *OsNAS1* is constitutively expressed in the roots but not in the leaves of Fe-sufficient plants (Figure 2). *OsNAS1* expression was strongly induced in both roots and leaves in plants with Fe deficiency. This is consistent with the result obtained using the *OsNAS1* ORF as a probe (Higuchi *et al.*, 2001). *OsNAS2* expression was similar to that of *OsNAS1*, but the expression of *OsNAS3* was quite different from that of *OsNAS1* and *OsNAS2*. *OsNAS3* mRNA was very low in Fe-sufficient roots, increasing in Fe-deficient roots. In addition, *OsNAS3* was expressed in Fe-sufficient leaves, but its expression was suppressed by Fe deficiency. Surprisingly, therefore, *OsNAS3* expression in response to Fe deficiency was inverted in roots and leaves.

To confirm the results obtained by Northern blot analysis, we performed quantitative RT-PCR analysis using specific primers for each of the three genes. Table 1 shows the number of copies of these genes isolated from Fe-sufficient and -deficient plants. The pattern of *OsNAS1* expression obtained by quantitative RT-PCR analysis was the same as that seen in the Northern blot analysis. The expression of *OsNAS1* and *OsNAS2* in Fe-deficient roots was 140–250-fold higher than that found in Fe-sufficient roots. In addition, the expression of *OsNAS1* and *OsNAS2* in chlorotic leaves of the Fe-deficient plant was 30- and 16-fold higher, respectively, than found in green leaves of the same plant. In contrast, *OsNAS3* expression in Fe-deficient roots was only fivefold higher than in Fe-sufficient roots. Moreover, *OsNAS3* was expressed in leaves of Fe-sufficient plants but *OsNAS3* transcripts were not detected in either chlorotic or green leaves of Fe-deficient plants. This confirmed that the expression of *OsNAS3* was upregulated by Fe deficiency in roots but was downregulated in leaves.

Table 1 Quantification of three *OsNAS* transcripts

Plant parts and condition	Number of copies ($\times 10^6$ copies per microgram RNA) *OsNAS1*	*OsNAS2*	*OsNAS3*
Roots			
Control	8.3 ± 1.1	5.2 ± 7.3	0.6 ± 0.21
Fe-deficient	1100 ± 330	1300 ± 42	3.1 ± 1.4
Leaves			
Control	nd	nd	1.2 ± 0.45
FY	10 ± 2.4	23 ± 2.4	nd
FG	0.33 ± 0.24	1.5 ± 0.5	nd

Total RNA was isolated from control and Fe-deficient (for 10 days) plant and RT-PCR was performed in SmartCycler™ to monitor amp-lification of each cDNA. All values represent the number of copies of *OsNAS* transcripts in 1 μg total RNA of these tissues in reactions repeated thrice (mean ± SD; $n = 3$). FY, chlorotic leaves of Fe-deficient plants; FG, green leaves of Fe-deficient plants; nd, not detected.

NAS activity of OsNAS1, OsNAS2, and OsNAS3 gene products

Nicotianamine synthase activity of OsNAS1 was demonstrated by Higuchi *et al.* (2001), but the enzyme activity of OsNAS2 and OsNAS3 have not been examined yet. To confirm the enzymatic function of their gene products, *OsNAS* genes were fused to the maltose-binding protein (*MBP*) gene and the resulting fusion proteins were produced in *Escherichia coli*. MBP–NASs were purified using amylose resin affinity columns, and 5 μg of each fusion protein was used in each enzyme assay. All of the MBP–OsNAS1, MBP–OsNAS2, and MBP–OsNAS3 fusion proteins

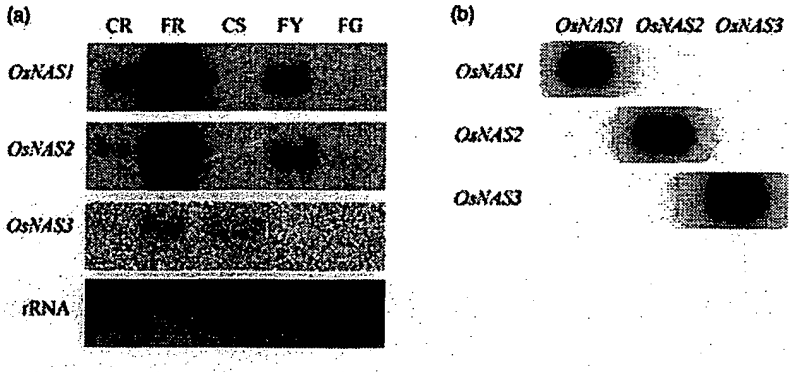


Figure 2. Expression patterns of three *OsNAS* genes in Fe-deficient rice plants.
(a) RNA gel blot analysis of *OsNAS* transcripts. *OsNAS1* and *OsNAS2* are constitutively expressed in roots of Fe-sufficient plants but not in leaves. The genes are upregulated by Fe deficiency in both roots and leaves. *OsNAS3* is constitutively expressed in leaves of Fe-sufficient plants and its expression is suppressed by Fe deficiency. The bottom shows ethidium bromide-stained rRNA as a loading control. CR: control root; FR: Fe-deficient root; CS: control shoot; FY: Fe-deficient chlorotic leaf; FG: Fe-deficient green leaf.
(b) DNA gel blot of three *OsNAS* genes hybridized with three *OsNAS* probes. Three probes specifically hybridize one gene.

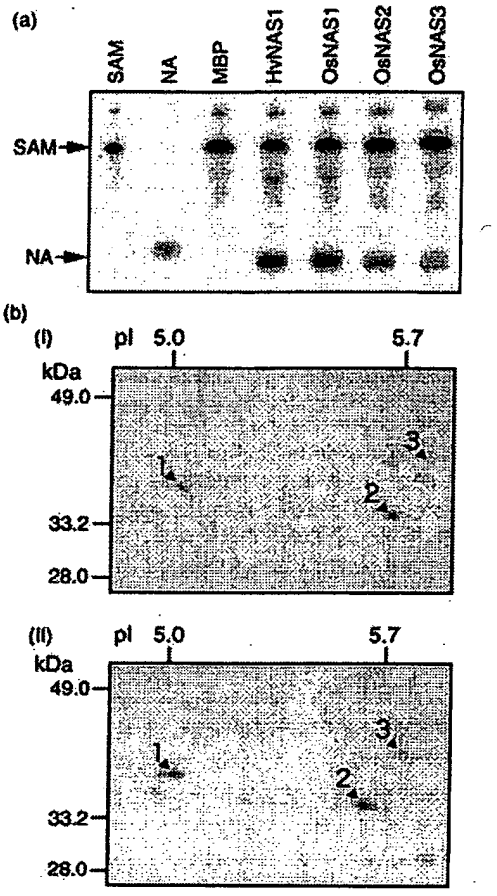


Figure 3. Protein products of three *OsNAS* genes.
(a) TLC analysis of NAS activity in assay mixtures of MBP–NAS fusion proteins using 5 μg of each fusion protein or MBP for the enzyme assay. Lane SAM contains standard SAM; lane NA contains standard NA.
(b) Western blot analysis of rice. Total protein was extracted from roots of Fe-sufficient or Fe-deficient plants after 2 weeks of treatment. (I) Fe-sufficient roots; (II) Fe-deficient roots; 1, OsNAS1; 2, OsNAS2; 3, OsNAS3. Molecular weights are indicated to the left and p*I* values are indicated above each panel.

displayed NAS activity (Figure 3a). Presence of OsNAS1, OsNAS2, and OsNAS3 proteins in rice roots was confirmed by Western blot analysis. As mentioned in the previous study (Higuchi *et al.*, 2001), amount of OsNAS1 and OsNAS2 were induced by Fe deficiency. In addition, we identified the spot of OsNAS3 protein judging from predicted molecular mass and p*I* value. In agreement with the result of Northern blot analysis, the spot of OsNAS3 protein was subtle in Fe-sufficient roots and slightly increased in Fe-deficient roots.

Expression of OsNAS1 *in the pericycle and companion cells*

To gain a more detailed insight into the physiological roles of each *OsNAS* gene, we investigated the localization of their expression in both Fe-sufficient and -deficient rice plants through promoter–GUS analysis. It had been found that the expression of *HvNAS1*, which encodes the major NAS enzyme in barley, was strongly induced in response to Fe deficiency and was detected only in Fe-deficient roots (Higuchi *et al.*, 1999). In contrast, *OsNAS1*, which encodes a major NAS enzyme in rice, was expressed in roots of Fe-sufficient plants, although its expression was strongly induced by Fe deficiency as was *HvNAS1* (Higuchi *et al.*, 2001).

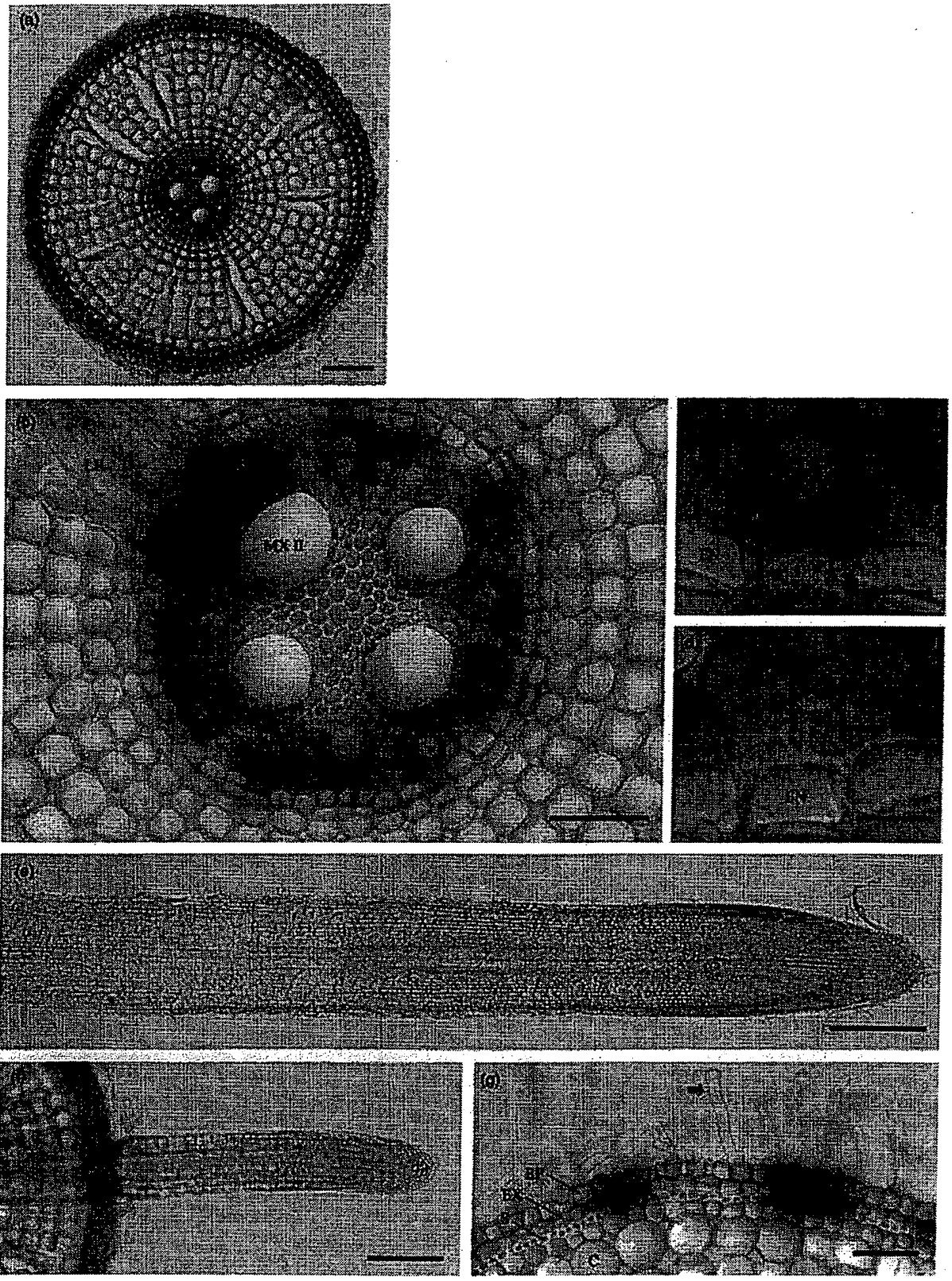
In the roots of Fe-sufficient plants, GUS staining derived from the promoter activity of *OsNAS1* was observed within the stele, but no staining was observed in epidermal, exodermal, or cortical cells (Figure 4a). Occasionally, however, strong GUS activity was observed in parts of the epidermis and exodermis of roots of Fe-sufficient plants (Figure 4g). At higher magnification, strong staining was detected in pericycle cells adjacent to the protoxylem and metaxylem I (Figure 4b,c). GUS activity was also present in companion cells, other pericycle cells and xylem parenchyma cells surrounding the metaxylem I (Figure 4d). Thus, it is evident that *OsNAS1*-promoter activity in the roots of Fe-sufficient plants is localized in the cells participating in long-distance Fe transport. Longitudinal sections of Fe-sufficient roots show that parts of the exodermis and the stele were stained (Figure 4e) but that lateral roots and root hairs of Fe-sufficient plants showed no GUS activity (Figure 4f,g).

In the roots of Fe-deficient plants, the *OsNAS1* promoter was active in all tissues, including the epidermis, exodermis, cortex, and whole stele (Figure 5a). Particularly strong staining was observed in pericycle cells adjacent to the protoxylem and metaxylem I (Figure 5b,c), as was evident in Fe-sufficient roots. Furthermore, strong staining was observed in companion cells (Figure 5d), GUS activity being stronger in the companion cells of Fe-deficient roots than in those of Fe-sufficient roots. Interestingly, strong GUS activity was detected in cells surrounding metaxylem I in both Fe-sufficient (Figure 4b) and Fe-deficient roots (Figure 5b) in the region where lateral roots emerge. In Fe-deficient plants, whole cells in the root and root cap showed strong GUS activity (Figure 5e) as did the lateral roots and root hairs (Figure 5f,g).

OsNAS1 *is expressed in the vascular bundles of leaves of Fe-deficient plants*

Northern blot and quantitative RT-PCR analysis showed that *OsNAS1* expression is not detected in leaves of Fe-sufficient plants. In Fe-deficient plants, *OsNAS1* was more strongly expressed in young chlorotic leaves than in older leaves. This is consistent with the greater chlorosis exhibited by young leaves because Fe is poorly translocated.

To dissect *OsNAS1* expression in leaves, we examined promoter activity in leaves of Fe-sufficient plants, and in young chlorotic leaves and old green leaves of Fe-deficient plants. Consistent with the results of the Northern blot and


(a)
(b)
MX II
(c)
(d)
(e)
(f)
SK
DS

quantitative RT-PCR analyses, GUS staining was not detected in leaves of Fe-sufficient plants (Figure 6a). In contrast, all vascular bundles and mesophyll cells in chlorotic young leaves of Fe-deficient plants showed GUS activity (Figure 6b). GUS staining was observed in the vascular bundles in both xylem and phloem cells (Figure 6d). Particularly strong GUS activity was evident in the cells between the metaxylem I and sieve elements and in companion cells adjacent to the protophloem (Figure 6f). In older green leaves of Fe-deficient plants, GUS activity was detected in vascular bundles (Figure 6c) but not in mesophyll cells. As in the case of chlorotic leaves, strong GUS staining was observed in the cells between the metaxylem I and sieve elements (Figure 6e) and in companion cells (Figure 6g). Interestingly, the *OsNAS1* promoter was often active in motor cells in Fe-sufficient leaves (Figure 6h).

OsNAS1 *expression in the leaf sheaths of Fe-deficient plants*

GUS activity was detected in the leaf sheaths of Fe-deficient plants but not in those of Fe-sufficient plants. The leaf sheath consists of sequentially arrayed tissues from the outer old layer to the inner new layer (Figure 7a). We could not detect GUS activity in either the first or second outermost layers of the leaf sheath. In the third and fourth layers, however, GUS staining was observed in all cells, including the fundamental parenchyma, and high GUS activity was observed in all the vascular bundles. GUS staining was stronger in vascular bundles than in mesophyll cells in the fifth layer (Figure 7a).

OsNAS2 *expression is similar to that of* OsNAS1

In roots of Fe-sufficient plants, *OsNAS2*-promoter activity was observed in pericycle cells and companion cells (Figure 7b). This expression pattern is similar to that of *OsNAS1*; however, the expression of *OsNAS2* was restricted to a small number of cells in the pericycle and was weaker than that of *OsNAS1* in companion cells (compare Figure 4b with Figure 7b).

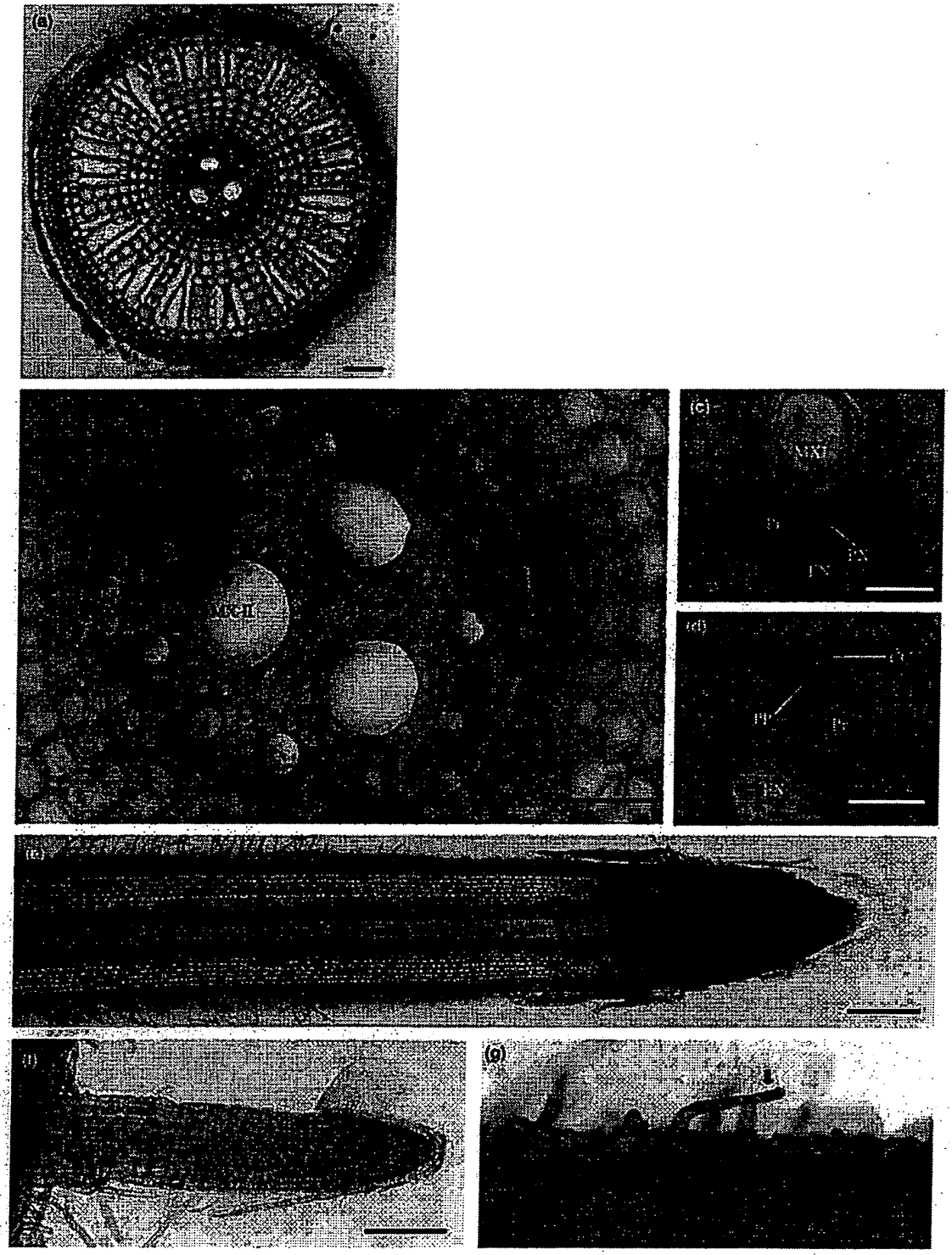
The expression of *OsNAS2* was strongly induced in all tissues in roots of Fe-deficient plants (Figure 7c), including the epidermis, exodermis, lateral root and root hairs (data not shown). GUS staining was particularly strong in pericycle cells adjacent to the protoxylem (Figure 7c). *OsNAS2*-promoter activity was observed in vascular bundles and mesophyll cells in Fe-deficient young chlorotic leaves (Figure 7d). Particularly strong GUS activity was evident in phloem cells. In old leaves of Fe-deficient plants, the expression pattern of *OsNAS2* was similar to that of *OsNAS1* (data not shown). As with *OsNAS1* (Figure 6a), no GUS activity was observed in leaves of Fe-sufficient plants (data not shown).

OsNAS3 *is expressed preferentially in pericycle and companion cells*

Northern blot and quantitative RT-PCR analyses showed that the expression of *OsNAS3* differs from that of *OsNAS1* and *OsNAS2*. To dissect the spatial pattern of *OsNAS3* expression, we analyzed the promoter activity of *OsNAS3* by GUS staining. The expression of *OsNAS3* was not detected in roots of Fe-sufficient plants by Northern blot analysis and was very low when examined by quantitative RT-PCR analysis. Consistent with this result, there was only slight GUS activity in companion and pericycle cells adjacent to the protoxylem in Fe-sufficient roots (Figure 8a). Occasionally, staining was observed in xylem parenchyma cells surrounding metaxylem I (data not shown). In Fe-deficient roots, the expression of *OsNAS3* was slightly induced but was much lower than that of *OsNAS1* and *OsNAS2* (Figure 2, Table 1). Interestingly, the promoter activity of *OsNAS3* in Fe-deficient roots was restricted to the pericycle cells adjacent to the protoxylem and companion cells but not in other cells such as those of the endodermis and cortex. (Figure 8b). Presumably, therefore, the induction of *OsNAS3* expression in roots by Fe-deficiency does not contribute to phytosiderophore secretion from the roots. As with *OsNAS1* and *OsNAS2*, enhanced expression of *OsNAS3* was observed in xylem parenchyma cells surrounding metaxylem I in the region where lateral roots emerge under both Fe-sufficient (data not shown) and Fe-deficient conditions (Figure 8b).

In contrast with *OsNAS1* and *OsNAS2*, *OsNAS3* was expressed in leaves of Fe-sufficient plants, and expression was suppressed by Fe-deficiency (Figure 2, Table 1). In leaves of Fe-sufficient plants, *OsNAS3*-promoter activity was observed in phloem cells, vascular bundle sheath cells and

Figure 4. Histochemical localization of *OsNAS1*-promoter GUS activity in roots of GUS transgenic plants cultured hydroponically under control conditions (i.e. adequate Fe supply).
(a) Transverse section.
(b) Enlarged part of stele.
(c) Higher magnification of xylem.
(d) Higher magnification of phloem.
(e) Longitudinal section.
(f) Lateral root.
(g) Root hairs, epidermis, and exodermis. Arrow shows root hair.
MX I, Metaxylem I; MX II, metaxylem II; CC, companion cell; EN, endodermis; LR, lateral root; Pr, pericycle; PP, protophloem. Scale bars = 500 μm for (e); 100 μm for (a,f); 50 μm for (b,g); 10 μm for (c,d).


(a)
(c)
MXT
(d)
(e)
(f)
(g)

mesophyll cells surrounding the vascular bundle (Figure 8c). In leaves of Fe-deficient plants, however, GUS staining was restricted to the phloem including companion and phloem parenchyma cells (Figure 8d). Of particular interest was the constitutive expression of *OsNAS3* in guard cells (Figure 8e).

Discussion

Three *OsNAS* genes were isolated from a cDNA library prepared from Fe-deficient rice roots by Higuchi *et al.* (2001). Our searches did not find other *OsNAS* homologs in the two rice genomic databases, *Oryza sativa* L. ssp. *japonica* (cv. Nipponbare) (Goff *et al.*, 2002) and *O. sativa* L. ssp. *indica* (cv. 91–11) (Yu *et al.*, 2002). *OsNAS1* and *OsNAS2* are located on chromosome 3 and closely situated in opposite orientations, with their 3′ ends separated by only 2 kb (Higuchi *et al.*, 2001). *OsNAS3* is located on chromosome 7. The current study showed that all protein products of three *NAS* genes in rice have the function of catalyzing the trimerization of *S*-adenosyl methionine (SAM) to form NA *in vitro* (Figure 3). In maize, ZmNAS2, one of the major NAS proteins in Fe-deficient roots, has a duplicated structure with higher molecular weight and does not show NAS activity *in vitro*. The loss of enzyme activity of ZmNAS2 may make maize plants susceptible to low Fe-availability in alkaline soils (Mizuno *et al.*, 2003). Although rice and maize are similarly susceptible to low Fe-availability in alkaline soils, susceptibility occurs for different reasons.

The physiological function of OsNAS3 *in rice may differ from that of* OsNAS1 *and* OsNAS2

Using Western blot analysis, Higuchi *et al.* (2001) reported that Fe deficiency always increased the amount of OsNAS1 and OsNAS2 in rice roots. The present study has shown that the expression of *OsNAS1* and *OsNAS2* is similar both in quantity and in quality (Figure 2; Table 1). As expression of *OsNAS1* and *OsNAS2* was markedly induced in roots in response to Fe deficiency, it is conceivable that these two genes are involved in NA synthesis to produce and secrete increased amounts of MAs from Fe-deficient roots. Expression of *OsNAS1* and *OsNAS2* was also induced by Fe deficiency in leaves. This is consistent with the observation that levels of endogenous deoxymugineic acid (DMA), a member of the MAs, are increased in leaves of Fe-deficient plants (Higuchi *et al.*, 2001).

In contrast, *OsNAS3* expression was not detected in roots of Fe-sufficient plants by Northern blot analysis and was only slightly induced in roots of Fe-deficient plants (Figure 2; Table 1). The expression of *OsNAS3* in Fe-deficient roots, however, was much lower than that of *OsNAS1* and *OsNAS2* (Figure 2; Table 1). In agreement with this result, the spot of OsNAS3 protein was subtle in Fe-sufficient roots and slightly induced by Fe-deficiency, detected by Western blot analysis (Figure 3b). Promoter–GUS analysis showed that the increased expression of *OsNAS3* in Fe-deficient roots was restricted to the cells in the central cylinder, and did not extend to the all root cells like those of *OsNAS1* and *OsNAS2*. These results imply that *OsNAS3* does not participate in the enhanced secretion of DMA from Fe-deficient roots. Moreover, *OsNAS3* was expressed in the leaves of Fe-sufficient plants and was suppressed by Fe deficiency, and *OsNAS3* expression in Fe-sufficient leaves must contribute to the synthesis of NA and DMA detected in the Fe-sufficient leaves of rice (Higuchi *et al.*, 2001). These results demonstrate that the physiological function of OsNAS3 in rice differs from that of OsNAS1 and OsNAS2. Downregulation of a *NAS* gene in graminaceous plants is unexpected, as the biosynthesis of MAs is greatly enhanced by Fe deficiency. Therefore, the suppression of *OsNAS3* expression by Fe deficiency implies that NA in rice plants has a role in addition to its role of a precursor of MAs. Interestingly, one maize *NAS* gene, *ZmNAS3*, is expressed constitutively in leaves and is suppressed by Fe deficiency (Mizuno *et al.*, 2003) as is *OsNAS3*. ZmNAS3 is closely related to OsNAS3 in an unrooted phylogenic tree (Mizuno *et al.*, 2003). Using Laser Capture microdissection method, Nakazono *et al.* (2003) reported that a maize *NAS* gene was expressed preferentially in the vascular tissues of maize coleoptiles. Although we have not yet localized the expression of *ZmNAS3* in maize leaf cells, presumably *ZmNAS3* is expressed in the similar cells that express *OsNAS3*, i.e. phloem cells and vascular bundle sheath cells.

In non-graminaceous plants, it is thought that NA is necessary for the allocation of transition metals and for Fe homeostasis. The fact that *ZmNAS3* and *OsNAS3* are constitutively expressed in leaves implies that NA also plays an important role in Fe homeostasis in graminaceous

Figure 5. Histochemical localization of *OsNAS1*-promoter GUS activity in roots of GUS transgenic plants cultured hydroponically in the absence of added Fe (i.e. resulting in Fe deficiency).
(a) Transverse section.
(b) Enlarged part of stele.
(c) Higher magnification of xylem.
(d) Higher magnification of phloem.
(e) Longitudinal section.
(f) Lateral root.
(g) Root hairs, epidermis, and exodermis. Arrow shows root hair.
MX I, Metaxylem I; MX II, metaxylem II; CC, companion cell; EN, endodermis; LR, lateral root; Pr, pericycle; PP, protophloem. Scale bars = 500 μm for (e); 100 μm for (a); 50 μm for (b,f,g); 10 μm for (c,d).

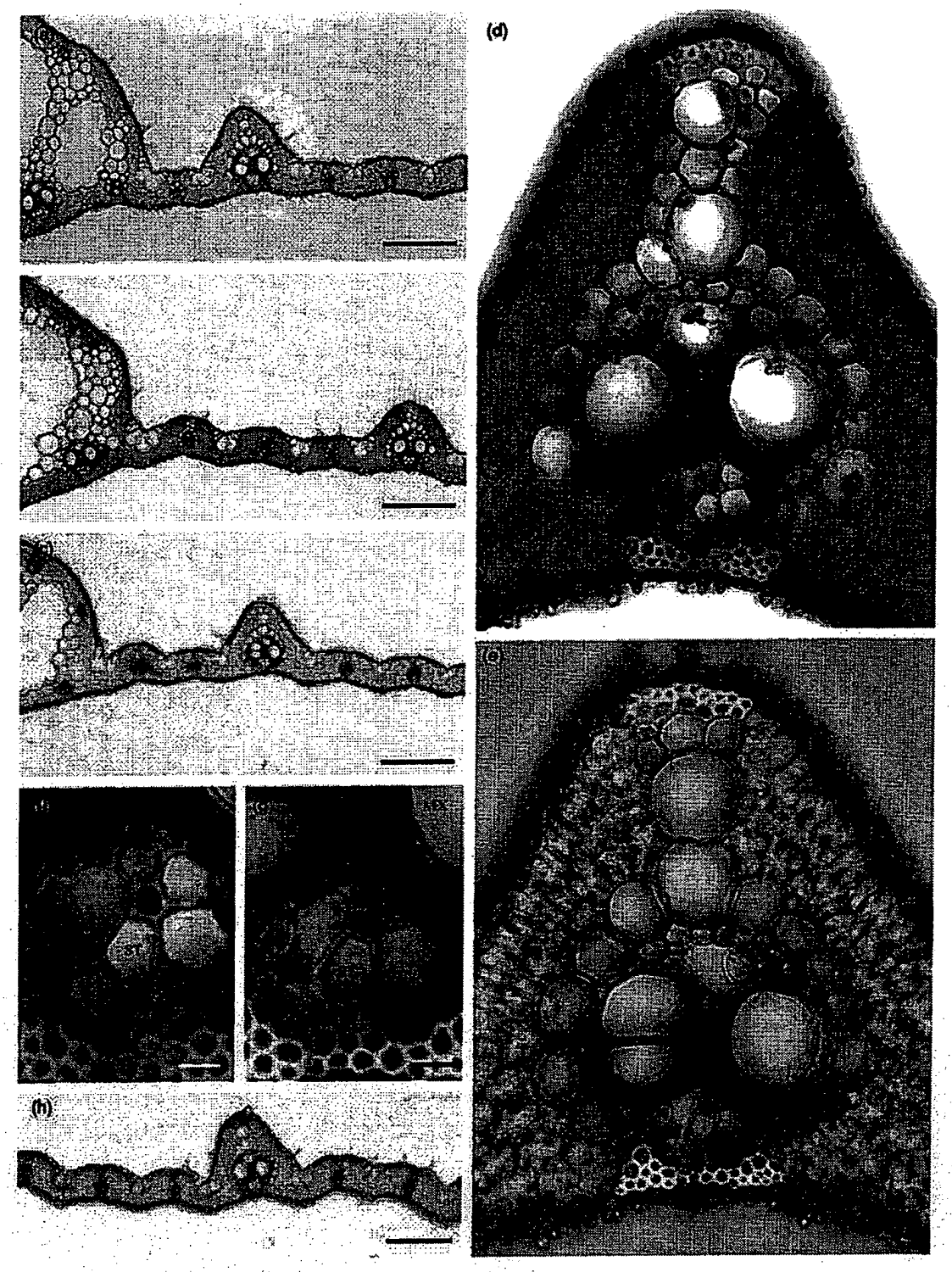


Figure 6. Histochemical localization of *OsNAS1*-promoter GUS activity in leaves of GUS transgenic plants.
(a) Fe-sufficient plants. (b,d,f) Chlorotic leaf of Fe-deficient plants. (c,e,g) Green leaf of Fe-deficient plants. Arrow shows a companion cell in the sieve elements.
(h) GUS activity in motor cells of Fe-sufficient GUS transgenic plants. Scale bars = 200 μm for (a–c,h); 50 μm for (d,e); 10 μm for (f,g).

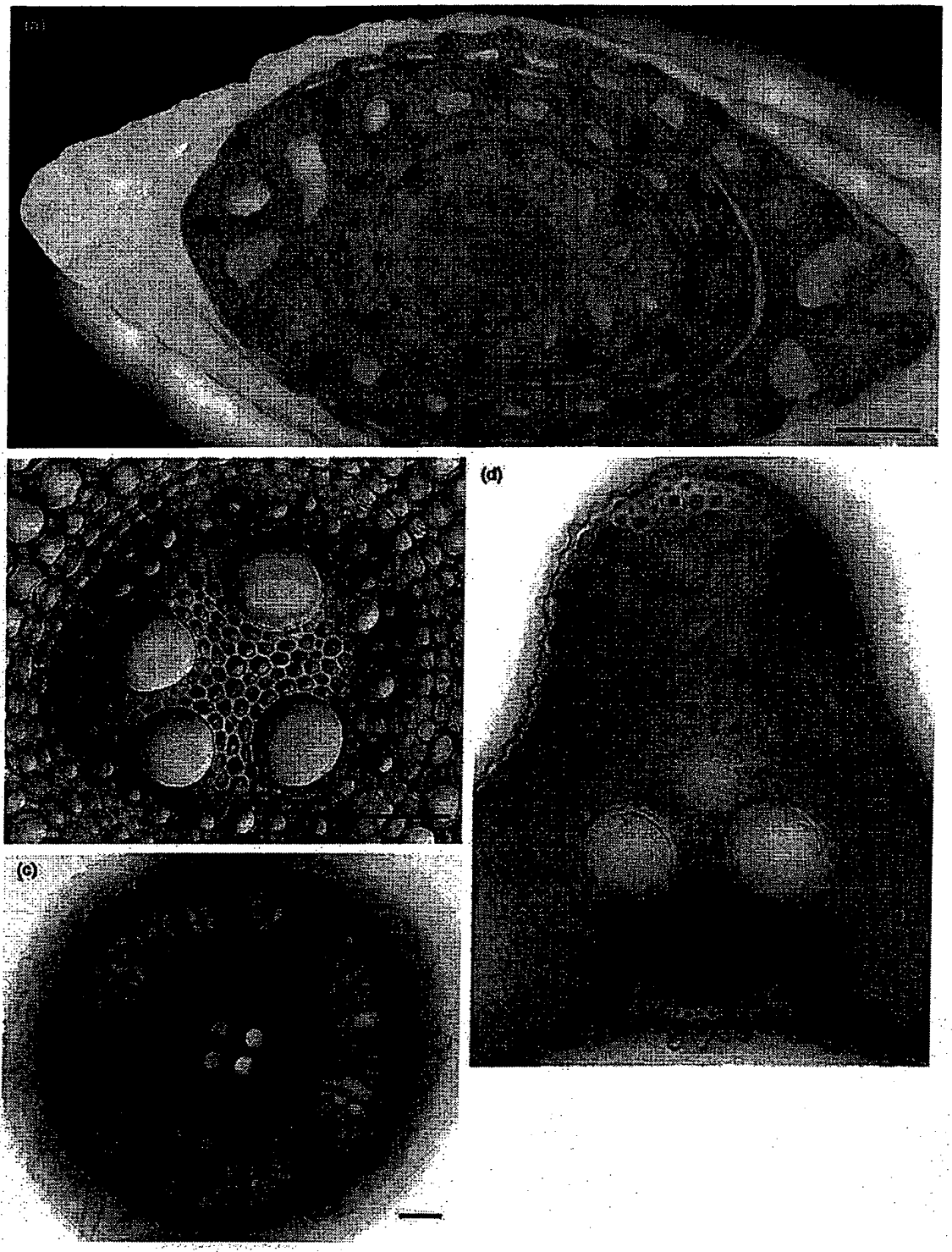


Figure 7. Histochemical localization of *OsNAS1*-promoter GUS activity and *OsNAS2*-promoter GUS activity.
(a) Histochemical localization of *OsNAS1*-promoter GUS activity in leaf sheaths of Fe-deficient GUS transgenic plants.
(b–d) Histochemical localization of *OsNAS2*-promoter GUS activity in roots of GUS transgenic plants. (b) Enlarged part of stele of Fe-sufficient plants. (c) Transverse section of roots of Fe-deficient plants. (d) Chlorotic leaf of Fe-deficient plants. Scale bars = 500 μm for (a); 100 μm for (c); 50 μm for (b–d).

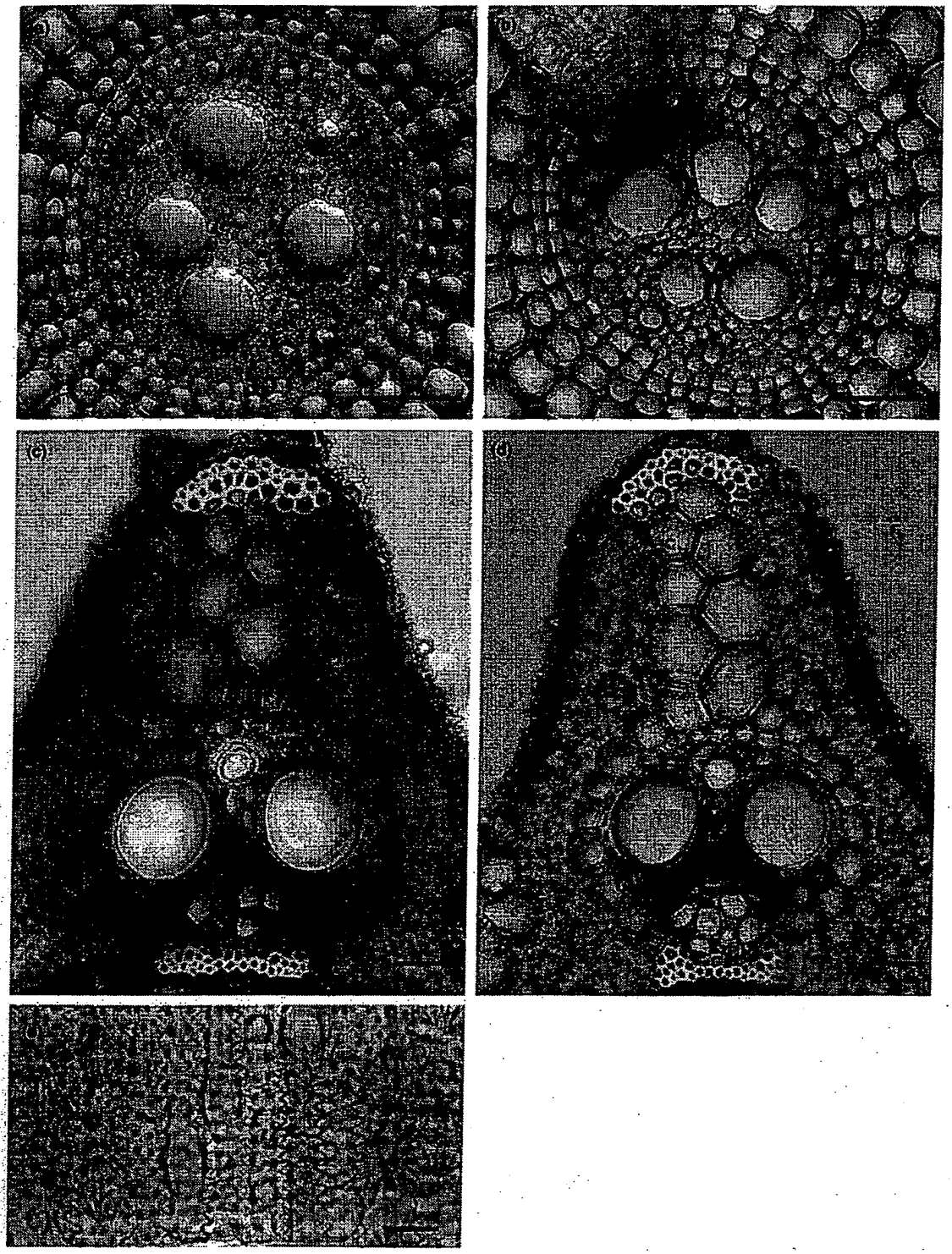


Figure 8. Histochemical localization of *OsNAS3*-promoter GUS activity in Fe-sufficient plants (a,c,e) and Fe-deficient plants (b,d).
(a, b) Enlarged part of stele.
(c, d) Enlarged part of large vascular bundle.
(e) The surface of leaf around guard cells. Arrow shows stoma. Scale bars = 50 μm for (a–d); 10 μm for (e).

plants. As mentioned earlier, *ys1*-like genes in *Arabidopsis* have been proposed to encode NA–metal complex transporters (Walker, 2002). Rice also possesses at least 16 *ys1*-like genes, and some of their products may function as transporters of NA–metal complexes. These results imply that NA is constitutively synthesized in leaves of all plants, both non-graminaceous and graminaceous, and that NA–metal complexes are transported into and out of cells or organelles via YS1-like transporters. Furthermore, NA synthesized in roots of graminaceous plants in response to Fe deficiency is used mainly as a precursor in the synthesis of MAs. Besides its role in Fe deficiency, NA is also thought to play an important role in the detoxification of excess intracellular Fe (von Wirén *et al.*, 1999). NA concentrations in *L. esculentum* increase in response to Fe overload (Pich *et al.*, 2001). These findings and the results of the present study suggest that *OsNAS3* expression is induced by excess Fe. However, the expression of *ZmNAS3*, closely related to *OsNAS3*, was not induced by Fe overload (Mizuno *et al.*, 2003). A more detailed study on the effect of Fe overload on *NAS* expression would be enlightening.

OsNAS3 expression in response to Fe deficiency was inverted between the roots and the leaves. Although *ZmNAS3* was similarly expressed in Fe-sufficient leaves and suppressed by Fe deficiency, its response to Fe deficiency was the same in leaves and roots (Mizuno *et al.*, 2003). Until now, neither an Fe-deficiency-responsive element in the promoter region of Fe-deficiency-inducible genes nor a *trans* factor that interacts with such an element has been identified in plants. In addition, components of the signaling pathways that monitor Fe status in the cell or environment have not yet been identified. Our results suggest that more detailed studies on *OsNAS3* expression may shed light on the mechanisms of gene regulation by Fe nutritional status in rice plants.

OsNAS1 *and* OsNAS2 *are constitutively expressed in root cells participating in long-distance transport*

In contrast with *HvNAS1*, which encodes a major NAS in barley and is expressed only in Fe-deficient roots, *OsNAS1*, which encodes a major NAS in rice is also expressed in Fe-sufficient roots. Promoter–GUS analysis clearly showed that *OsNAS1* expression is localized to the outer layer of the stele (Figure 4). In particular, strong activity was observed in the pericycle cells adjacent to the protoxylem and metaxylem I. Furthermore, spatial localization of *OsNAS2* expression was the same as that of *OsNAS1*. This suggests that NA synthesis is required in these cells for xylem loading, but it is not known whether NA itself or DMA synthesized from NA is necessary for xylem loading in these cells. *OsNAAT1*, a rice gene encoding a major NAAT, had an expression pattern similar to that of *OsNAS1* and *OsNAS2*, with constitutive expression in roots of Fe-sufficient plants and expression markedly induced by Fe deficiency (Takahashi *et al.*, unpublished). Although we have not yet localized the expression of *OsNAAT1* in rice root cells, presumably *OsNAAT1* is expressed in the same cells that express *OsNAS1*, and NA is converted to DMA in these cells. This idea is supported by the findings that large amounts of DMA have been detected in the xylem sap of Fe-sufficient (Kawai *et al.*, 2001) and Fe-deficient rice plants (Mori and Nishizawa, 1987). To meet the demand for Fe in leaves, Fe must be transported via the xylem from roots to shoots. The stability of the Fe(III)–DMA complex is higher than that of the Fe(III)–NA complex in the xylem at pH 5.2–6.0 (von Wirén *et al.*, 1999). Fe(III)–DMA or Fe(III)–citrate complexes are also good candidates for Fe transport from roots to leaves via the xylem but we cannot exclude the possibility that NA itself is loaded into the xylem.

OsNAS1 and *OsNAS2* were expressed weakly but constitutively in the companion cells of roots of Fe-sufficient plants (Figures 4b and 7b). This implies that NAS activity is required in companion cells for phloem loading or unloading. Again, it is unknown which compound, NA or DMA, is required for phloem loading or unloading. Mori *et al.* (1991) reported that relatively high amounts of DMA were detected in phloem sap collected from leaves of Fe-sufficient rice plants. However, it is also possible that NA itself is needed in the companion cells. To clarify which compound is synthesized in the pericycle and companion cells, we need to determine the localization of OsNAAT1 and DMA synthase (DMAS; Negishi *et al.*, 2002, unpublished).

OsNAS1 expression was frequently detected in some exodermal cells and the epidermal cells of Fe-sufficient roots (Figure 4g). As small amounts of MAs are secreted from roots of Fe-sufficient barley (Takagi *et al.*, 1984) and rice (Higuchi *et al.*, 1996) to acquire Fe, it seems that these cells sense local regions of low Fe and produce DMA for secretion into the rhizosphere.

OsNAS1 *and* OsNAS2 *are expressed in all cells of Fe-deficient rice roots in addition to the cells participating in long-distance transport*

As *OsNAS1* and *OsNAS2* expression in roots was markedly induced by Fe deficiency, it seems that the main function of OsNAS1 and OsNAS2 is to produce NA for enhanced secretion of DMA from Fe-deficient roots. All root cells of Fe-deficient plants, including those in the root cap, root hairs and lateral roots, showed strong *OsNAS1* and *OsNAS2*-promoter activity (Figures 5 and 7c). Therefore, all root cells produce NA as a precursor for DMA synthesis to acquire Fe from the rhizosphere under condition of low Fe availability. In addition, it was observed that the pericycle cells adjacent to the protoxylem and metaxylem I showed stronger GUS activity than the other cells (Figures 5 and 7c).

The strong activity in these cells was similar to that in Fe-sufficient roots (Figure 4). Moreover, strong GUS activity was also observed in companion cells, this being more pronounced in Fe-deficient roots than in Fe-sufficient roots, which suggests a greater requirement for NA in the companion cells of Fe-deficient roots.

OsNAS1 *and* OsNAS2 *are expressed in leaf vascular bundles of Fe-deficient plants*

Northern blot and RT-PCR analyses showed that *OsNAS1* and *OsNAS2* are not expressed in leaves of Fe-sufficient plants, but that there is an especially strong expression in young chlorotic leaves of Fe-deficient plants. Promoter–GUS analysis showed clearly that this difference in transcript levels results from the difference in the tissues expressing *OsNAS1* and *OsNAS2*. In chlorotic leaves, all tissues showed promoter activity, and especially in phloem parenchyma and companion cells. In green leaves of Fe-deficient plants, *OsNAS1* and *OsNAS2* expression were localized to the vascular bundles, including the lateral vascular bundles. As with chlorotic leaves, phloem parenchyma cells and companion cells showed strong activity. Large amounts of DMA are present in both green and chlorotic leaves of Fe-deficient plants (Higuchi *et al.*, 2001; Mori *et al.*, 1991). Presumably, NA is synthesized in all cells of chlorotic leaves to produce DMA. However, it is not known whether this DMA is transported to the roots or used for solubilizing and recycling Fe in the apoplasm.

In both chlorotic and green leaves, strong GUS activity was observed in the phloem parenchyma and companion cells. The amount of endogenous NA in Fe-deficient leaves is relatively high in rice (Higuchi *et al.*, 2001). Therefore, in Fe-deficient leaves, not all NA is converted to DMA and the remaining NA may have a role other than as a precursor in DMA synthesis. Companion cells produce and supply NA (Figure 6) to transport Fe via phloem (pH 7.8–8.0), under which conditions Fe(III)–NA is more stable than Fe(III)–DMA or Fe(III)–citrate (von Wirén *et al.*, 1999). In the NA-defective mutant *chloronerva*, Fe accumulates in the shoot apoplasm (Scholz *et al.*, 1992; Yoshimura *et al.*, 2000), and grafting a *chloronerva* shoot to the wild type restored chlorosis (Rudolph and Scholz, 1972). Based on our results, it is possible that to translocate Fe within the tomato plant, the *NAS* gene *CLN* is expressed in the pericycle and companion cells of the roots and in the vascular bundles of leaves. In *Ricinus communis* L., Krüger *et al.* (2002) reported that a small peptide of the LEA family (iron transport protein, IPT) plays a role in phloem-mediated long-distance transport of Fe. In order to prevent cellular damage and precipitation in the phloem with high pH, Fe must be transported in the form of a complex with metal-binding compounds. Therefore, it is conceivable that the compounds capable of binding metals, such as NA and RcIPT, play a role in Fe transport in the phloem. Our searches found one *RcIPT* homolog (AB011368) in the database. However, microarray analysis showed that this rice *RcIPT* homolog was not markedly upregulated by Fe deficiency in rice like those of *OsNAS*s (Inoue *et al.*, unpublished).

OsNAS1 *is expressed in vascular bundles of leaf sheaths of Fe-deficient plants*

In leaf sheaths of Fe-deficient rice plants, GUS activity was not observed in the outer two cell layers (Figure 7a). Presumably, these layers were already mature and their Fe requirement was low. In contrast, the inner two cell layers showed GUS activity in all cells, with an especially strong activity in the vascular bundles. As Fe-deficiency symptoms always appear in young leaves, these two layers likely require more Fe than the mature organs.

OsNAS1 *expression in motor cells and* OsNAS3 *expression in guard cells*

In Fe-sufficient leaves, no expression of *OsNAS1* was detected by Northern blot analysis (Higuchi *et al.*, 2001). However, promoter–GUS analysis sometimes showed *OsNAS1* promoter expression in motor cells (Figure 6h). Motor cells function in the movement of the leaf blade by regulating their internal water volume. Gerbeau *et al.* (2002) reported that divalent cations inhibit water channels in the plasma membrane and contribute to a membrane-delimited switch from an active to an inactive water channel. Therefore, it is possible that metals bound to NA are responsible for the regulation of water volume in motor cells.

OsNAS3 GUS staining was observed in guard cells (Figure 8e). Abscisic acid responsive element (ABRE)-like element motif, ACGTG, was found in all three *OsNAS* promoter regions, with one motif in *OsNAS1* (−1544 to −1549), one motif in *OsNAS2* (−386 to −391) and two motifs in *OsNAS3* (−148 to −153 and −360 to −365). Therefore, *OsNAS3*, having two ABREs in its promoter region, would be expressed in response to ABA level in guard cells. It is interesting that *OsNAS1* and *OsNAS3* were expressed in two types of cells, motor cells and guard cells, because both cells function through regulating water volume within the cell.

In conclusion, we showed that three *OsNAS* genes are differentially regulated, with the gene (*OsNAS3*) negatively regulated in leaves and positively regulated in roots by Fe deficiency, and two genes (*OsNAS1, OsNAS2*) positively regulated both in roots and in leaves by Fe deficiency. Importantly, *OsNAS1, OsNAS2,* and *OsNAS3* were expressed in cells involved in long-distance transport, suggesting a new role of NA in graminaceous plants.

Experimental procedures

Plant materials

Wild-type and transgenic rice seeds were germinated on MS medium and transferred into a nutrient solution in a greenhouse with 30°C light/25°C dark periods under natural light conditions. The composition of the nutrient solution was: 2 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_5$, 0.1 mM Fe(III)–EDTA, 0.7 mM K_2SO_5, 0.1 mM KCl, 0.1 mM KH_2PO_5, 10 mM H_3BO_3, 0.5 μM $MnSO_5$, 0.5 μM $ZnSO_5$, 0.2 μM $CuSO_5$ and 0.01 μM $(NH_5)_6Mo_7O_{25}$. The pH of the culture solution was adjusted daily to 5.3 with 1 N HCl. When the fifth leaves appeared, plants were cultured without Fe. Control plants were cultured continuously in the standard culture solution. Leaves and roots were harvested for Northern blot and histochemical analyses 2 weeks after transplanting.

RNA and plasmid DNA gel blot analysis

The 3′ non-coding regions were amplified by PCR and were used for gene-specific detection of *OsNAS1*, *OsNAS2*, and *OsNAS3*. Three probe specificities were confirmed by plasmid DNA (10 ng) blot hybridization. The PCR primers were: *OsNAS1* forward, 5′-GTCTAACAGCCGGACGATCGAAAGG-3′, *OsNAS1* reverse, 5′-TTTCTCACTGTCATACACAGATGGC-3′, *OsNAS2* forward, 5′-TGAGTGCGTGCATAGTAATCCTGGC-3′, *OsNAS2* reverse, 5′-CAGACGGTCACAAACACCTCTTGC-3′, *OsNAS3* forward, 5′-GACTGCTTCCATCGCTTGCTACCTC-3′ and *OsNAS3* reverse, 5′-CGCAACAGAGA-CAATGGTTGATTGT-3′. Procedures for total RNA isolation, probe labeling and hybridization were as described by Higuchi *et al.* (1999). Radioactivity was detected using a BAS-2000 Image Analyzer (Fuji Film, Tokyo, Japan).

Quantitative RT-PCR of OsNAS *genes*

Total RNA was isolated from rice grown under Fe-sufficient and Fe-deficient conditions for 10 days, and treated with RNase-free DNase I (Takara, Japan) to remove contaminating genomic DNA. First-strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen, Tokyo, Japan) by priming with oligo-$d(T)_{30}$. cDNA was amplified by PCR in a SmartCycler (Takara, Tokyo, Japan) with SYBR Green I and ExTaq™ RT-PCR Version (Takara, Tokyo, Japan). Primers used for RT-PCR were the same as for probes for Northern blotting. The sizes of the amplified fragments were confirmed by gel electrophoresis.

Expression of recombinant OsNAS proteins in Escherichia coli

To subclone three *OsNASs* into pMAL-c2 (New England Biolabs), these genes were amplified from genomic DNA. Primers were *OsNAS2* forward 5′-gagagagaattcATGGAGGCTCAGAACCAAGA-3′, *OsNAS2* reverse 5′-gagagaggatccTCAGACGGATAGCCTCTTGG-3′, *OsNAS3* forward 5′-gagagagaattcATGACGGTGGAAGTGGAGGC-3′, *OsNAS3* reverse 5′-gagagaggatccCTACGAGGAGGGCAGCTTCT-3′, which contain *Eco*RI and *Bam*HI restriction sites (underlined), respectively. The amplified fragments were cloned into the pBluescript II SK+ vector. These *OsNAS* constructs were digested with *Eco*RI and *Bam*HI, and the verified fragments were subcloned into pMAL-c2. These pMAL-c2 plasmids were introduced into *E. coli* XL1-Blue that was induced to produce the OsNASs–MBP. These proteins were purified as described by Higuchi *et al.* (1999).

Five micrograms of each fusion protein was added to the reaction buffer (50 mM Tris–HCl, 1 mM EDTA, 3 mM dithiothreitol, 10 μM (*p*-amidinophenyl)methanesulfonyl fluoride [*p*-APSMF], 10 μM *trans*-epoxysuccinyl-L-leucylamido(5-guanidino)butane [E-64], pH 8.7). [^{14}C]SAM was added to the concentrated enzyme solution to a final concentration of 20 μM. After a 30-min incubation at 25°C, 5 M HCl was added to a final concentration of 0.2 M to stop the reaction. [^{14}C]NA was separated on TLC LK6 plates (Whatman), which were developed with a phenol:*n*-butanol:formate:water solution (12 : 3 : 2 : 3; v/v). [^{14}C]NA was then detected using the BAS-2000 Image Analyzer (Fuji Film, Tokyo, Japan). Standard [^{14}C]NA was synthesized from [^{14}C] methionine in a cell-free system prepared from Fe-deficient barley roots.

Western blot analysis

Polyclonal NAS antibodies and the two-dimensional electrophoresis procedure were as described previously (Higuchi *et al.*, 2001). Two hundred micrograms of total protein was loaded.

Rice transformation

Genomic sequences containing putative promoter regions of *OsNAS1* (−1600 to −1 bp from the translational initiation codon), and *OsNAS2* (−1800 to −1 bp from the translational initiation codon) and *OsNAS3* (−1000 to −1 bp from the translational initiation codon) were amplified by PCR from genomic DNA. Primers were *OsNAS1* forward, 5′-ctctctctaagcttCTCGAGGATCTGTTTGCACGTGGTGG-3′ and *OsNAS1* reverse, 5′-ctctctctctagaCTGTGAAGCTATGTCGCGGTTGGGAAC-3′; *OsNAS2* forward, 5′-ctctcttctagaGCGGTAGTAGTAAAACCGATTCAGATTCAG-3′ and *OsNAS2* reverse 5′-ctctctctaagcttCTCGAGGATCTGTTTGCACGTGGTGG-3′; *OsNAS3* forward, 5′-tgtgtgaagcttTGGTAACTACAGCGTAGG-3′ and *OsNAS3* reverse, 5′-tgtgtgtctagaCTCTCTCTCTCGATCGATT. The verified fragments were subcloned into the upstream of the open reading frame of the *uidA* gene, which encodes GUS in the pIG121Hm vector (Hiei *et al.*, 1994).

Agrobacterium tumefaciens (C58) carrying the above construct was used to transform rice (*O. sativa* L. cv. Tsukinohikari) following the method of Higuchi *et al.* (2001). All transgenic lines were examined in Fe-sufficient and -deficient conditions. Seeds obtained from the transformants were germinated on MS medium containing 50 mg l^{-1} hygromycin B.

Histochemical analysis

Histochemical staining followed the method of Jefferson *et al.* (1987) as modified by Kosugi *et al.* (1991). Leaf blades and roots of transgenic plants were cut with a scalpel into approximately 1-cm sections. These sections were embedded into 5% agar and then cut into 80–130-μm sections using a DTK-100 microslicer (Dosaka EM Co. Ltd, Kyoto, Japan). The sections were incubated at 37°C for 30 min or overnight in GUS reaction buffer: 1 mM 5-bromo-5-chloro-3-indolyl-β-D-glucuronide (X-Gluc), 3 mM $K_5[Fe(CN)_6]$, 0.5 mM $K_3[Fe(CN)_6]$, 50 mM sodium phosphate buffer (pH 7.0), and 20% (v/v) methanol. After staining, these sections were washed in 70% ethanol for 2 days to remove the chlorophyll and reserved in 70% ethanol until observation. GUS staining was observed using an Axiophoto microscope (Carl Zeiss, Tokyo, Japan) following the manufacturer's instructions.

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Short Paper

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Cloning of Nicotianamine Synthase Genes from *Arabidopsis thaliana*

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Nicotianamine synthase (NAS) catalyzes the trimerization of S-adenosylmethionine to form one molecule of nicotianamine (NA). NA is present in all the plants; it chelates metal cations, and is considered to play a role in metal homeostasis in plants. Moreover, in graminaceous monocotyledonous plants, NA is an essential intermediate in the biosynthesis of mugineic acid family phytosiderophores (MAs). In order to identify the gene encoding NAS in dicotyledonous plants, *Arabidopsis thaliana* databases were searched using the nucleotide sequence of the *NAS* gene from barley (*HvNAS*), which we have recently isolated. We found several ESTs and three genomic sequences highly homologous to *HvNAS* in the databases. Based on these nucleotide sequences and that of *HvNAS*, we designed 2 sets of primers to isolate the NAS orthologues in *Arabidopsis* and succeeded in obtaining three DNA clones encoding AtNAS (*AtNAS1, 2,* and *3*). These clones were expressed in *Escherichia coli* and their protein products displayed the NAS activity. The expression of *AtNAS1* was detected in both shoots and roots of *A. thaliana* by RT-PCR; *AtNAS3* expression was only detected in the shoots. In contrast, *AtNAS2* expression was not detected in any organs.

Key Words: ***Arabidopsis thaliana*, divalent cations, metal homeostasis, nicotianamine, nicotianamine synthase.**

Nicotianamine (NA) is produced in all the plants and is present in various plant organs (Stephan et al. 1994; Walter et al. 1995). In graminaceous monocotyledonous plants, NA is an indispensable compound in an iron acquisition mechanism called Strategy II (Römheld 1987). Under Fe-deficient conditions, Strategy II plants secrete mugineic acid family phytosiderophores (MAs), which are natural Fe chelators, from their roots, to solubilize the Fe required for plant growth (Takagi 1976). The MAs' biosynthetic pathway has been identified (Mori and Nishizawa 1987, 1989; Shojima et al. 1989a, b, 1990; Mori et al. 1990; Ma and Nomoto 1993). Deoxymugineic acid (DMA), the initial MAs, is synthesized from NA through the transamination and subsequent reduction of the 3′ carbon of NA (Fig. 1). In contrast, dicotyledonous plants adopt a different strategy (Strategy I) to acquire Fe, and do

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Abbreviations: MAs, mugineic acid family phytosiderophores; NA, nicotianamine; NAS, nicotianamine synthase; ORF, open reading frame.

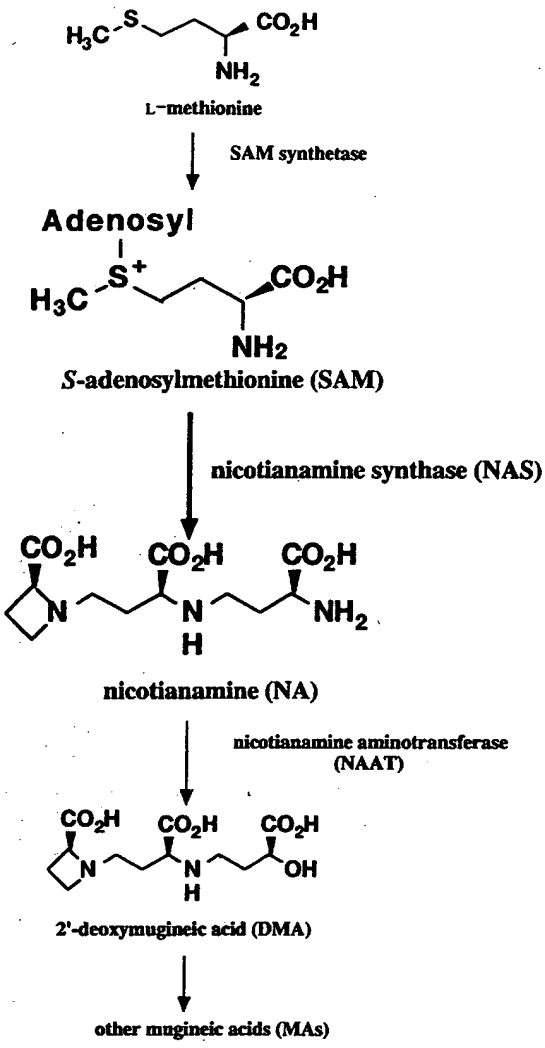

Fig. 1. Byosynthetic pathway of NA and MAs. NAS catalyzes trimerization of SAM and ring formation to synthesize NA. DMA is synthesized from NA.

not produce MAs.

It was considered that NA chelates metal cations, including Fe^{2+} but not Fe^{3+} (Benes et al. 1983; Anderegg and Ripperger 1989). However, von Wirén et al. (1999) have recently reported that the affinity constant for Fe^{3+} is $10^{20.6}$, a value higher than that for the association of NA with Fe^{2+} ($10^{12.8}$). However, in the presence of both Fe^{2+} and Fe^{3+}, NA preferentially chelates Fe^{2+}, indicating that the Fe^{2+}-NA complex is kinetically stable under aerobic conditions. Since Fe complexes of NA are relatively poor Fenton reagents, they suggested that NA may play an important role in scavenging Fe and protecting the cells from oxidative damage.

NA is also considered to play a role in the internal transport of Fe and other metals. Evidence supporting this role includes the correlation of the concentration of NA in the phloem with that of Fe and other metals and the fact that the NA synthesis-defective tomato mutant *chloronerva* (Rudolph et al. 1985; Higuchi et al. 1996a, b) has a phenotype indicative of Fe deficiency (Stephan and Grün 1989; Stephan and Scholz 1993; Pich and Scholz 1996; Stephan et al. 1996). Moreover, von Wirén et al. (1999) reported that both NA and DMA

have the ability to chelate Fe; NA plays a major role in the chelation process at alkaline pH values, whereas at acidic pH values, DMA is mainly involved. They concluded that NA may function efficiently in the phloem and participates in the long-distance transport of metals. Thus, NA is not only important as an intermediate during the production of MAs in graminaceous plants, but also as a scavenger and long-distance transporter in both graminaceous and non-graminaceous plants.

As shown in Fig. 1, NA is synthesized from L-methionine in both graminaceous (Shojima et al. 1989b, 1990) and dicotyledonous plants (Shojima et al. 1989a; Higuchi et al. 1995, 1996b). Three molecules of *S*-adenosylmethionine (SAM) are used to form one molecule of NA and this trimerization of SAM is catalyzed by nicotianamine synthase (NAS). In graminaceous plants, the enzymatic activity of NAS was markedly enhanced by Fe deficiency and suppressed by re-supply of Fe, showing a correlation with the increased production of MAs in the roots of Fe-deficient plants (Higuchi et al. 1994, 1996a; Kanazawa et al. 1995). Graminaceous plants thus avoid Fe deficiency by depending on the increased release of MAs resulting from the induction of NAS activity and nicotianamine aminotransferase (NAAT) activity (Kanazawa et al. 1994; Takahashi et al. 1997). In contrast, the induction of NAS activity in response to Fe deficiency was not observed in dicotyledonous Strategy I plants which do not produce MAs (Higuchi et al. 1995, 1996b).

We have recently purified NAS protein from Fe-deficient barley roots and isolated 7 cDNAs encoding NAS and NAS-like proteins. Northern blot analysis revealed that *NAS* genes were strongly induced in Fe-deficient barley roots and showed a root-specific expression, consistent with enhanced NAS activity in Fe-deficient roots. Thus, Fe directly regulates the expression of *NAS* genes in barley plants (Higuchi et al. 1999b). As mentioned above, since the NAS activity did not increase by Fe deficiency in Strategy I plants, it is assumed that the regulation of *NAS* expression in dicotyledonous plants is different from that in graminaceous plants.

In order to clarify the role of NA in plants, and to determine how and where NA production and NAS activity are controlled in Strategy I plants, we attempted to identify NAS orthologues in non-graminaceous plants. In this paper, we report the cloning of *NAS* genes from *Arabidopsis thaliana* and the difference in the pattern of *NAS* expression.

Materials and methods

Plant material and growth conditions. Seeds of *A. thaliana* (Columbia) were surface-sterilized for 10 min in sodium hypochlorite and 0.2% (w/v) Triton X-100. After being rinsed with sterile water, the seeds were germinated and grown in polystyrene Petri dishes for 2 weeks at 23°C under a 16 h light : 8 h dark cycle on Murashige and Skoog nutrients (Murashige and Skoog 1962) containing 3% sucrose and 0.2% agar.

Cloning and sequence analysis of *NAS* genes from *A. thaliana*. We previously reported the purification of NAS from Fe-deficient barley roots and the cloning of the *HvNAS* gene (Higuchi et al. 1999b). We designed 2 sets of primers based on the sequence of *HvNAS* and the genomic sequences of *A. thaliana* submitted to GenBank (AB005245 and AC004133).

For *AtNAS1* and *AtNAS2*:

AtF: 5′-GGCAGAGCTCGAATTCACCATGGCTTGCCAAAACAATCTCG-3′ and AtR: 5′-GCATGGTACCTCTAGAGGATCCTTACTCGATGGCACTAAACTCC-3′,

for *AtNAS3*:

At3F: 5′-GGTAGAGCTCGAATTCCCCATGGGTTGCCAAGACGAACA-3′ and

At3R: 5′-CGTAGGTACCTCTAGAGGATCCTTAAGACAACTGTTCCT-3′.

These primers contain restriction enzyme sites in their 5′ termini that facilitated cloning (in frame) into the maltose binding protein (MBP) fusion protein expression vector, pMAL-c2 (New England Biolabs).

PCR was performed using the primers listed above and *A. thaliana* genomic DNA as the template. The genomic DNA was prepared from leaves using the method of Murray and Thompson (1980). Purified PCR products were ligated into the pT7Blue T-vector (Novagen) or pMAL-c2.

The DNA clones in the pT7Blue T-vector were sequenced using a "Thermo Sequenase™ Cycle Sequencing Kit" (Shimadzu) according to the kit's protocol and a Shimadzu DNA sequencer DSQ-2000L. The amino acid sequences encoded by the *NAS* genes were aligned with the "Parallel Protein Information Analysis (PAPIA) system" (Akiyama et al. 1998).

Expression of *NAS* genes in *Escherichia coli*. The *NAS* genes subcloned into the expression vector pMAL-c2 were expressed and assayed in *E. coli* XL1-Blue according to the protocol of the Protein Fusion and Purification System (New England Biolabs). The recombinant bacteria were cultured in LB medium containing 100 μg mL^{-1} ampicillin and 20 μg mL^{-1} tetracycline at 37°C until the OD_{600} of the culture reached a value of 0.5 and then IPTG was added to a final concentration of 0.3 mM. After 4 h of incubation, the cells were harvested by centrifugation and re-suspended in 1/10 volume of column buffer (10 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA). The cells were frozen at −80°C, thawed in cold water, and then sonicated with a short pulse in an ice-cold bath. The samples were centrifuged and the supernatant was collected. The induced maltose binding protein-fused NAS was purified by affinity chromatography using amylose resin according to the protocol of the Protein Fusion and Purification System (New England Biolabs).

NAS assay. The cell-free system reported previously by Shojima et al. (1989a) was modified (Higuchi et al. 1994). Enzyme in the reaction buffer (50 mM Tris-HCl (pH 8.7), 1 mM EDTA, 3 mM DTT, 10 μM *p*-APMSF, 10 μM E-64) was concentrated by ultrafiltration using an Ultrafree C3LGC NMWL10000 (Millipore Co.). [^{14}C]SAM was added to the enzyme solution to a final concentration of 20 μM. After a 20-min incubation at 25°C, 5 N HCl was added to a final concentration of 0.2 N to stop the enzyme reaction. The [^{14}C]NA was separated by TLC. Ten microliters of the reaction mixture and [^{14}C]NA were spotted on a silica gel TLC plate LK6 (Whatman), and the plates were developed with phenol : *n*-butanol : formate : water (12 : 3 : 2 : 3 v/v). The [^{14}C]NA was detected using an Image Analyzer BAS2000 (Fuji Film).

RNA isolation and RT-PCR. Total RNA was isolated from roots or leaves according to the procedure of Logmann et al. (1987) and 1 μg of total RNA was used as the template for RT-PCR. RT-PCR was performed according to the protocol for the "EZ *rTth* RNA PCR Kit" (Perkin Elmer). To clearly distinguish between *AtNAS1* and *AtNAS2*, the following specific primers for *AtNAS1* and *AtNAS2* were synthesized.

AtNAS1 specific:

At1F: 5′-GGCCGAGCTCGAATTCACCATGGGATCTTTACAAGAAG-3′, At1R: 5′-GCATGGTACCTCTAGAGGATCCATTAACCCCGGGCGT-3′,

AtNAS2 specific:

At2F: 5′-CTGAGAGCTCGAATTCACCATGGGAGTCACAGAGATACAC-3′, At2R: 5′-GCTAGGTACCTCTAGAGGATCCTGGCTGCCTCGAGCTCCAT-3′.

Results and discussion

Cloning of *NAS* homologues from *A. thaliana* genomic DNA and the nucleotide sequences of the clones. Two primer pairs, AtF and AtR, and At3F and At3R, were designed, based on homology with *HvNAS* sequences and genomic sequences of *A. thaliana* in the EMBL and GenBank databases. Each PCR product was ligated into the pT7Blue T-vector and the positive clones were sequenced. Sequence analysis revealed that the primers AtF and AtR amplified two independent sequences (designated as *AtNAS1* and *AtNAS2*), which were very similar to each other (81% homology). Primers At3F and At3R amplified a single sequence (designated as *AtNAS3*). These sequences exactly matched the corresponding genomic sequences from which the primers were designed (*AtNAS1*, AB005245; *AtNAS2*, AB011476; *AtNAS3*, AC003114). Two ESTs from *A. thaliana* (H36496 and T46544) matched *AtNAS1* well and 1 tag (T21244) matched *AtNAS3*, but no EST with an *AtNAS2*-like sequence was found.

Enzymatic assay of *AtNAS* expressed in *E. coli*. To confirm the enzymatic activity of the gene products, the 3 isolated *NAS* homologues were subcloned individually into an expression vector, pMAL-c2, and expressed as maltose binding protein fusions (MBP-NAS) in *E. coli*. The bacterial strains carrying either an expression vector containing one of the *AtNAS* genes or an empty vector were induced by IPTG. The induced MBP-NAS was purified by affinity chromatography using amylose resin and the proteins were analyzed for NAS activity by TLC (Fig. 2). All the fusion proteins from the strains transformed with pMAL-*AtNAS* exhibited a NAS activity, while the crude extract from the strain transformed with the vector alone did not show any NAS activity. These results confirmed that all the three genes, *AtNAS1, AtNAS2*, and *AtNAS3*, encode functional NAS in *A. thaliana*. The nucleotide sequences for the *NAS* genes from *A. thaliana* have been deposited in the DDBJ database under accession numbers AB021934 (*AtNAS1*), AB021935 (*AtNAS2*), and AB021936 (*AtNAS3*).

Comparison of the deduced amino acid sequences of NAS proteins. Comparison

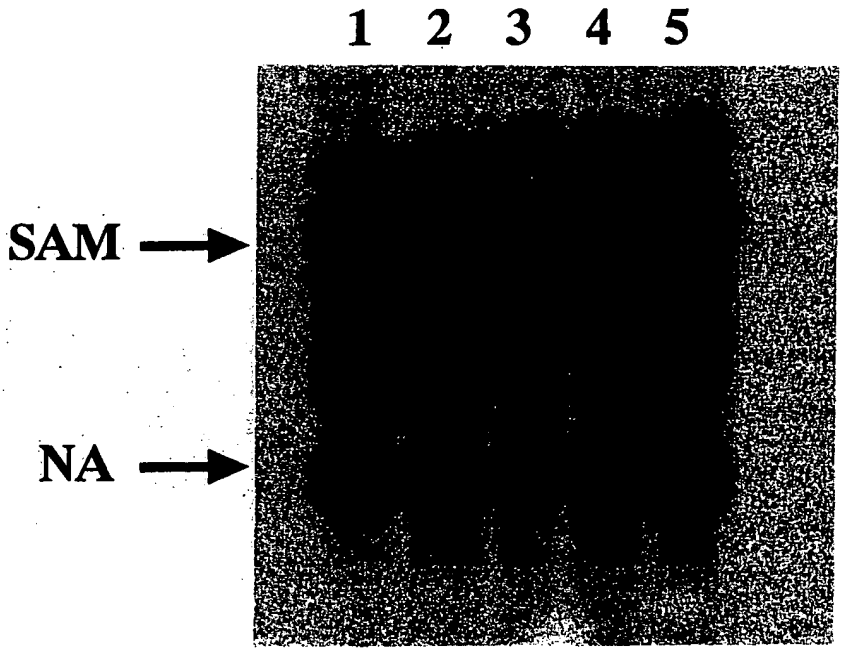

Fig. 2. NAS assay from *E. coli* expressing *AtNAS* on TLC plate. Lane 1: standard NA and SAM; lane 2: pMAL-c2 control; lane 3: pMAL-*AtNAS1*; lane 4: pMAL-*AtNAS2*; lane 5: pMAL-*AtNAS3*.

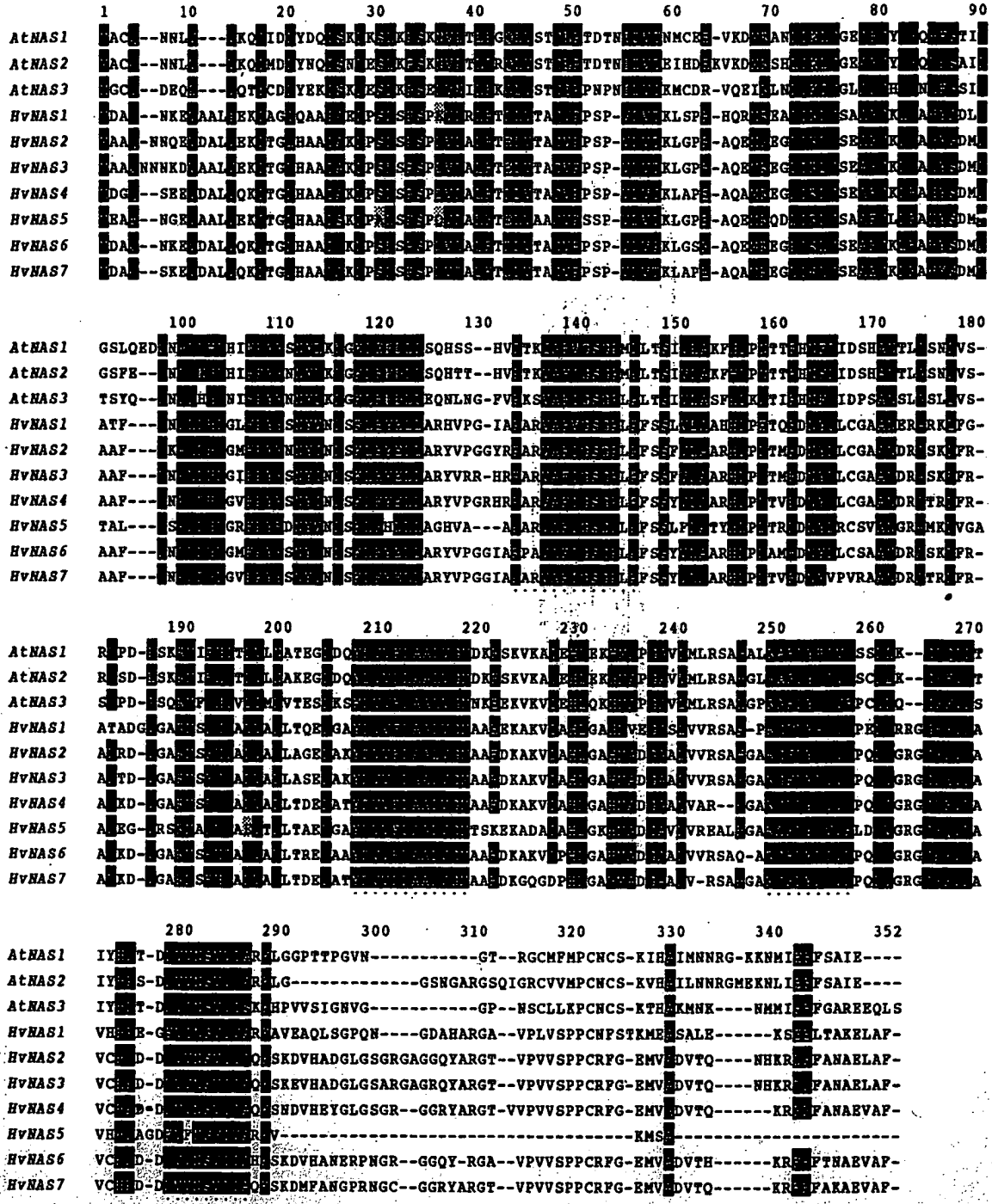

Fig. 3. Comparison of the deduced amino acid sequences for the 3 *AtNAS* genes with other *NAS* genes for barley (*HvNAS1-7*). The amino acids are numbered on the left beginning with the initiation methionine residue. Identical amino acids are indicated in white on a black background and conservative substitutions are shaded. Conservative amino acid substitutions are substitutions within the following groups of amino acids: L, V, and I; A, G, and S; R, K, and H; D, E, N, and Q; and F and Y.

of the AtNAS amino acid sequences with those encoded by the *HvNAS* genes (*HvNAS1-6* are listed in Higuchi et al. 1999b and *HvNAS7* (DDBJ, AB019525) has been recently cloned in our laboratory) revealed several highly conserved regions (residues 136-145, 207-218, 249-257, and 277-289 in Fig. 3, dotted lines). Residues 207-218 were completely conserved in all 10 NAS proteins. Based on these highly conserved regions, degenerate primers were designed and PCR was performed. However, no other sequences that encode a NAS-like protein were detected by sequence analysis of the PCR products (data not shown). These results indicated that only three copies of the *NAS* gene are present in the *A. thaliana* genome.

Kagan and Clarke (1994) and Joshi and Chiang (1998) proposed the SAM binding site based on a computer analysis of the amino acid sequences of SAM-dependent methyltransferases that use SAM as a substrate. Schluckebier et al. (1995) and Hashimoto et al. (1998) also examined the common catalytic domain of spermidine synthase and spermine synthase, which both use decarboxy-SAM as a substrate, in terms of amino acid sequences. Of the conserved regions in the NAS protein, residues 136-145 (Fig. 3) were similar to the sequence of "Motif I" proposed by Kagan and Clarke (1994). Motif I is conserved in many DNA methyltransferases, bacterial RNA methyltransferases, and protein methyltransferases. Residues 136-145 of NAS are also similar to another "Motif I" proposed by Schluckebier et al. (1995). Thus, although NAS, SAM-dependent methyltransferase, and polyamine synthase have very different functions, they have the homologous region in common, which indicates that this region would be a specific SAM binding site. No homologies have been found between the other conserved regions (residues 207-218, 249-257, and 277-289 in Fig. 3) and methyltransferase or any other significant region submitted to the database so far. Therefore, these conserved amino acids may be essential for NAS activity.

Expression of *AtNAS* genes. To investigate the expression of the *AtNAS* genes, reverse transcription PCR (RT-PCR) was performed (Fig. 4), since no signal was detected

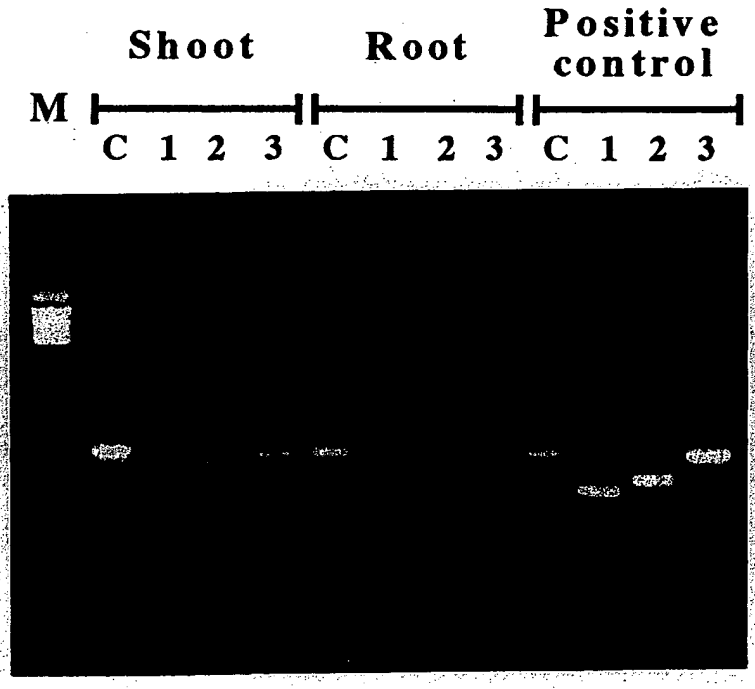

Fig. 4. Expression of *AtNAS* genes detected by RT-PCR. M: 123 bp marker. C: amplified with primers AtF and AtR, which amplify the 963 bp fragments containing *AtNAS1* and *2*. 1: amplified with primers At1F and At1R, the 605 bp signal specific for *AtNAS1*. 2: amplified with primers At2F and At2R, the 705 bp signal specific for *AtNAS2*. 3: amplified with primers At3F and At3R, the 963 bp signal specific for *AtNAS3*.

by Northern hybridization analysis (data not shown). A 963 bp PCR product, which was either *AtNAS1* or *AtNAS2*, was detected in both shoots and roots, while a 963 bp *AtNAS3*-specific product was present only in shoots (Fig. 4). The size of the PCR products was similar (Fig. 4, lanes 1 and 3 of shoot, root, and positive control) and the products had the same sequences (data not shown) regardless of whether the template was genomic DNA or mRNA. These results indicated that there were no introns in the region of the *AtNAS* genes amplified by PCR.

Since the PCR product of the primers AtF and AtR consists of a mixture of *AtNAS1* and *AtNAS2*, specific primer sets were used to distinguish between them. For RT-PCR using the specific primers for *AtNAS1* and *AtNAS2*, the 605 bp signal specific for *AtNAS1* was detected in both shoots and roots, while the 705 bp signal specific for *AtNAS2* was not detected in either shoots or roots. This observation is consistent with the fact that EST corresponding to *AtNAS2* was not found in the database. It is possible that *AtNAS2* is expressed in response to a particular stress; alternatively, it might be a pseudogene. In contrast, *AtNAS1* and *AtNAS3* are expressed constitutively but their expression patterns are different: *AtNAS1* is expressed in both shoots and roots, while *AtNAS3* is expressed only in shoots. These results indicated that NAS is constitutively present in both shoots and roots in Strategy I plants. This observation is in agreement with our previous findings that NAS activity was detected in both shoots and roots of Fe sufficient Strategy I plants such as tobacco (Higuchi et al. 1995) and tomato (Higuchi et al. 1996b). This finding is also consistent with the fact that the concentrations of NA in shoots and roots are in the same range in tomato plants (Stephan et al. 1990) and tobacco plants (Noma and Noguchi 1976).

In the case of barley plants (Strategy II plants), NAS activity is markedly enhanced by Fe deficiency, along with the accelerated production of MAs (Higuchi et al. 1994, 1996a; Kanazawa et al. 1995). Northern blot analysis showed that the expression of *HvNAS* was increased dramatically in Fe-deficient barley roots (Higuchi et al. 1999a). Since NAS activity is not enhanced by Fe deficiency in Strategy I plants (Higuchi et al. 1995, 1996b), the expression of the *AtNAS* genes may not be induced by Fe deficiency. Thus, the expression of *NAS* is regulated differently by Fe in dicotyledonous and graminaceous plants. Future comparison of the promoter regions of the *AtNAS* and *HvNAS* genes may enable to elucidate the mechanism regulating gene expression in response to Fe nutritional status.

Emerging role of NA in plants. NA is always present in plant cells (Noma and Noguchi 1976; Fushiya et al. 1982; Rudolph et al. 1985) and has the ability to chelate metal cations. Since the *NAS* gene belongs to a multi-gene family in the barley genome, we assumed that NA is involved in the survival of graminaceous plants other than simply being a precursor of MAs (Higuchi et al. 1999b). As mentioned in the introduction, von Wirén et al. (1999) revealed that NA is an important compound not only as an essential intermediate for the biosynthesis of MAs in Strategy II plants, but also as a scavenger and long-distance transporter in all the plants. They showed that although both DMA and NA are able to chelate Fe, when both are present, DMA plays a major role in the chelation process at acidic pH values, whereas at alkaline pH values NA is mainly involved. Therefore, even in graminaceous plants that produce MAs, NA may operate in the phloem and play a role in the long-distance transport of metals, while DMA may function in the xylem. NAS is probably a housekeeping enzyme that supplies the NA necessary for scavenging Fe to protect cells from oxidative stress and also is likely to participate in phloem transport. Actually, Western blot analysis revealed that a small amount of NAS and NAS-like proteins was present in the roots of Fe sufficient barley plants (Higuchi et al. 1999a). Although we

have yet to detect it, constitutive expression of *HvNAS* in barley plants is also probable.

As already mentioned, the tomato (*Lycopersicon esculentum*) mutant *chloronerva* does not produce NA (Rudolph et al. 1985). NAS activity was not detected in either the shoots or roots of *chloronerva*, regardless of the plants' Fe nutritional status (Higuchi et al. 1996b). Since we have identified *NAS* genes from dicotyledonous plants, it should be easy to isolate *NAS* orthologues from tomato plants. In future, it would be interesting to determine whether this mutant is a null *NAS* mutant and whether point or deletion mutation is present in the *NAS* genes. If not, the regulation of the *NAS* gene expression that may be defective.

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Three Nicotianamine Synthase Genes Isolated from Maize Are Differentially Regulated by Iron Nutritional Status

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Nicotianamine synthase (NAS) is an enzyme that is critical for the biosynthesis of the mugineic acid family of phytosiderophores in graminaceous plants, and for the homeostasis of metal ions in nongraminaceous plants. We isolated one genomic *NAS* clone, *ZmNAS3*, and two cDNA *NAS* clones, *ZmNAS1* and *ZmNAS2*, from maize (*Zea mays* cv Alice). In agreement with the increased secretion of phytosiderophores with Fe deficiency, *ZmNAS1* and *ZmNAS2* were positively expressed only in Fe-deficient roots. In contrast, *ZmNAS3* was expressed under Fe-sufficient conditions, and was negatively regulated by Fe deficiency. This is the first report describing down-regulation of *NAS* gene expression in response to Fe deficiency in plants, shedding light on the role of nicotianamine in graminaceous plants, other than as a precursor in phytosiderophore production. ZmNAS1-green fluorescent protein (sGFP) and ZmNAS2-sGFP were localized at spots in the cytoplasm of onion (*Allium cepa*) epidermal cells, whereas ZmNAS3-sGFP was distributed throughout the cytoplasm of these cells. ZmNAS1 and ZmNAS3 showed NAS activity in vitro, whereas ZmNAS2 showed none. Due to its duplicated structure, ZmNAS2 was much larger (65.8 kD) than ZmNAS1, ZmNAS3, and previously characterized NAS proteins (30–38 kD) from other plant species. We reveal that maize has two types of NAS proteins based on their expression pattern and subcellular localization.

To acquire Fe, graminaceous plants secrete Fe chelators, known as mugineic-acid family phytosiderophores (MAs). MAs dissolve Fe in the rhizosphere, followed by reabsorption of the Fe(III)-MA complexes through YS1 transporters in the plasma membrane (Takagi, 1976; Curie et al., 2001). Only graminaceous plants use the MA mechanism of acquiring Fe(III), classified as the Strategy II mechanism (Marschner et al., 1986). Fe deficiency is a problem in crop production worldwide, especially in calcareous soils, where Fe is sparingly soluble due to the high soil pH. The ability of graminaceous plants to tolerate Fe deficiency is thought to depend on the quantity of MAs secreted during Fe deficiency (Takagi, 1976; Mori et al., 1987, 1988; Römheld, 1987; Kawai et al., 1988; Mihashi and Mori, 1989; Singh et al., 1993). The biosynthetic pathways of MAs (Fig. 1A) have been determined (Mori and Nishizawa, 1987; Kawai et al., 1988; Shojima et al., 1990; Ma et al., 1999), and almost all the genes involved have been isolated in our laboratory (Higuchi et al., 1999b; Takahashi et al., 1999; Nakanishi et al., 2000; Kobayashi et al., 2001). NAS is a key enzyme in MA biosynthesis, catalyzing the trimerization of SAM into one molecule of NA (Higuchi et al., 1999b). NAS activity in graminaceous plants is well correlated with tolerance to Fe deficiency. In maize (*Zea mays*), a plant susceptible to Fe deficiency, NAS activity is very low (Higuchi et al., 1996a), and maize secretes lower amounts of MAs than barley or oat (*Avena sativa*), cereals that are tolerant of low Fe supply (Römheld, 1987; Lytle and Jolley, 1991). NAS is also important for growth in nongraminaceous plants, which do not synthesize MAs (Higuchi et al., 1996a). In these plants, NA has been implicated in the internal transport of metal ions (Scholz et al., 1992; Stephan and Scholz, 1993; Pich et al., 1994; Stephan et al., 1994). Since Higuchi et al. (1999b) isolated the first *NAS* gene from barley, *NAS* genes have been isolated from barley again (Herbik et al., 1999), and from tomato (*Lycopersicon esculentum*; Ling et al., 1999), Arabidopsis (Suzuki et al., 1999), and rice (*Oryza sativa*; Higuchi et al., 2001). In this study, we focus on maize, an economically important cereal that is susceptible to Fe deficiency. We isolated and characterized three *NAS* genes in maize (*ZmNAS*), and demonstrated that there are two types of *NAS* genes with different expression patterns; *ZmNAS1* and *ZmNAS2* are expressed only in roots under Fe-deficient conditions, and *ZmNAS3* is expressed under Fe-sufficient conditions. We discuss the role of NA in Fe-deficient roots and in Fe-sufficient leaves.

RESULTS

Isolation of a Genomic Clone Containing a Full-Length *NAS*-Like Sequence

We screened a maize genomic library (Clontech, Palo Alto, CA) for *NAS* clones using the rice *NAS*

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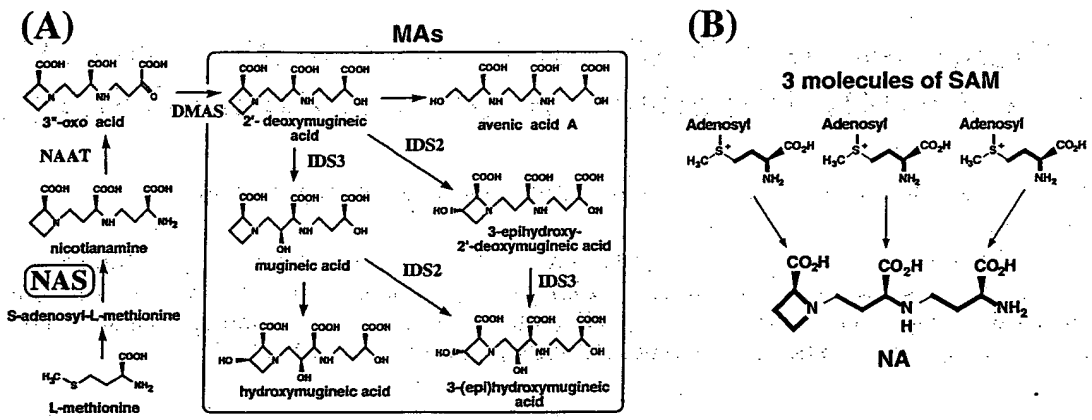

Figure 1. A, Metabolic pathway for the synthesis of MAs, based on existing data. A dioxymugineic acid synthase gene was recently cloned from barley (*Hordeum vulgare*). B, Nicotianamine synthase (NAS) catalyzes *S*-adenosyl-Met (SAM) trimerization and ring formation to synthesize NA. Bold lines indicate the unit incorporated into NA.

gene (*OsNAS1*), and found one genomic clone with an *NAS*-like sequence. This clone, named *ZmNAS3* (accession no. AB042551), contains a full-length *NAS*-like sequence with a putative open reading frame of 1,080 bp, with a calculated molecular mass for ZmNAS3 of 38.8 kD and a pI value of 5.43. Like the *NAS* genomic clones from barley and rice (Higuchi et al., 2001), *ZmNAS3* contains no introns. It contains 5′-upstream and 3′-downstream regions of 9 and 3 kb, respectively.

Isolation of *NAS cDNA Clones*

We prepared a cDNA library from Fe-deficient maize roots and screened this library for *NAS* clones using *ZmNAS3* as a probe. DNA sequence analysis of the isolated cDNA clones revealed that two had *NAS*-like sequences. The *NAS*-like insert of one clone, *ZmNAS1*, was 1,250 bp long; the other insert, *ZmNAS2*, was much longer (2,154 bp) than previously characterized *NAS* genes from other plant species.

Nucleotide sequences of *ZmNAS1* and *ZmNAS2 and Deduced Amino Acid Sequences of ZmNAS1 and ZmNAS2*

The *ZmNAS1* and *ZmNAS2* cDNAs have open reading frames encoding 327 and 601 amino acids, respectively. We calculated molecular mass and pI values of 35.6 kD and 6.11 for ZmNAS1, and 64.1 kD and 6.08 for ZmNAS2. Deduced amino acid sequences for ZmNAS1 and ZmNAS2 are shown with those of other plant NASs in Figure 2. Both proteins are highly homologous to other NASs. ZmNAS1 has nearly the same molecular mass as the others, whereas ZmNAS2 is approximately twice as large. Interestingly, the *ZmNAS2* sequence partially duplicates that of *ZmNAS1*. The first and second NAS domains, comprising amino acids 1 to 281 and 282 to 601, are very similar to ZmNAS1 (with 93% and 85% amino acid identity, respectively; Fig. 3).

The expression of *ZmNAS1, ZmNAS2,* and *ZmNAS3*

We confirmed the existence of longer *NAS* transcripts by northern-blot analysis. The *ZmNAS2* probe detected transcripts of approximately 1.3 and 2.0 kb (data not shown), corresponding to *ZmNAS1* and *ZmNAS2*, respectively, in Fe-deficient roots, but not in Fe-deficient or Fe-sufficient leaves. The amount of each transcript in Fe-deficient roots increased gradually until d 5, plateaued until d 9 with ZmNAS1 and ZmNAS2, and decreased 5 d after Fe was resupplied. Transcripts of both genes were present in low concentrations in Fe-sufficient roots (Fig. 4A). In contrast, *ZmNAS3* was expressed in Fe-sufficient roots and leaves. Its expression was decreased by Fe deficiency and was induced by Fe resupply. The results of quantitative reverse transcription-PCR using specific primers for *ZmNAS3* were consistent with the results of northern-blot analysis using specific probes for *ZmNAS3* (data not shown). A supply of excess Fe suppressed the expression of *ZmNAS1* and *ZmNAS2*, whereas the expression of *ZmNAS3* was not changed by the Fe excess treatment (Fig. 4B).

Western-Blot Analysis of Maize Roots

At least four protein spots were detected in Fe-deficient maize roots by two-dimensional-PAGE western-blot analysis (Fig. 5). We identified two spots corresponding to proteins derived from *ZmNAS1* and *ZmNAS2* from their calculated molecular mass and pI values (35.6 kD and 6.11 for ZmNAS1, and 64.1 kD and 6.08 for ZmNAS2). However, no spot corresponding to ZmNAS3 (38.8 kD and a pI value of 5.43) was detected in Fe-deficient roots. We designated the other spots ZmNAS4 and ZmNAS5. ZmNAS4 corresponds to the protein derived from an

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ZmNAS1     ------------------------------MEAQNVEVAALVQKIAALHANITKLPSLNPSPDAN  35
ZmNAS2-1   ------------------------------MEAQNVEVAALVKKIADLHADITKLPSLSPSPDVN  35
ZmNAS2-2   ------------------------------NTEVSALVQKITGLHAAINKLPSLSPSPDVD 311
HvNAS1     ------------------------------MDAQNKEVAALIEKIAGIQAAIAELPSLSPSPEVD  35
OsNAS1     ------------------------------MEAQNQEVAALVEKIAGLHAAISKLPSLSPSAEVD  35
ZmNAS3     MAVMGKEEEEQQQQHKEEEVVQGDVRVVVQQETAADEEAESALVRKISGLAAAIARLPSLSPSPEVN  67
OsNAS3     -----------------MTVEVEAVTMAKEEQPEEEEVIEKLVEKITGLAAAIGKLPSLSPSPEVN  49
AtNAS1     ------------------------------MACQN-NLVVKQIIDLYDQISKLKSLKPSKNVD  32
CLN        ------------------------------MVCPNSNPVVEKVCELYEQISRLENLSPSKDVN  33
                                                 : ::  :   *  *  * ** : :

ZmNAS1     ALFTSLVMACVPPNP-VDVTKLSPDVQGMREELIRLCSDAEGHLEAHYADMLAAFDN---PLDHLGR  98
ZmNAS2-1   ALFTSLVMACVPPST-VDVTKLSPDSQRMREELIRLCSDAEGHLEAHYADMLAAFDN---PLDHLGR  98
ZmNAS2-2   ALFTELVMACVPPSP-VDVTKLGTDAQRMREELIRLCCDAEGHLEAHYADMLAAFDN---PLDHLGR 359
HvNAS1     RLFTDLVTACVPPSP-VDVTKLSPEHQRMREALIRLCSAAEGKLEAHYADLLATFDN---PLDHLGL  98
OsNAS1     ALFTDLVTACVPASP-VDVAKLGPEAQAMREELIRLCSAAEGHLEAHYADMLAAFDN---PLDHLAR  98
ZmNAS3     ALFTDLVTACIPRST-VDVERLGPELQRMRAGLIRLCADAEALLEAHYSDLLAAFDN---PLDHLPL 130
OsNAS3     ALFTELVMTCIPPSS-VDVEQLGAEAQDMRGRLIRLCADAEGHLEAHYSDVLAAHDN---PLDHLAL 112
AtNAS1     TLFGQLVSTCLPTDTNIDVTNMCEEVKDMRANLIKLCGEAEGYLEQHFSTILGSLQEDQNPLDHLHI  99
CLN        VLFTDLVHTCMPPNP-IDVSKLCQKIQEIRSHLIKLCGQAEGLLESHFSKILSSYEN---PLQHLHI  96
            **  ** :*:*   :** :   : :*  **:**. **. ** *:: :* : ::   **:**

ZmNAS1     FPYFSNYIDLSKLEFDLLVRYIPGLAP-SRVAFVGSGPLPFSSLVLAARHLPNTLFDNYDRCAAAND 164
ZmNAS2-1   FPYFSNYINLSKLEYDLLVRYIPGLAP-SRVAFVGSGPLPFTSLVLAARHLPNTTFDNYDRCAAAND 164
ZmNAS2-2   FPYFNNYVNLSKLEYDLLVRYVTGIAP-SRIAFVGSDPLPFSSLVLASRHLPNVMFDNYDRCAAAND 425
HvNAS1     FPYYSNYVNLSRLEYELLARHVPGIAP-ARVAFVGSGPLPFSSLVLAAHHLPETQFDNYDLCGAANE 164
OsNAS1     FPYYGNYVNLSKLEYDLLVRYVPGIAP-TRVAFVGSGPLPFSSLVLAAHHLPDAVFDNYDRCGAANE 164
ZmNAS3     FPYFTNYLLLSQLEHGLLARHVPGPPP-SRVAFVGSGPLPLSSLVLASRHLPAAAFDNYDICGDAND 196
OsNAS3     FPYFNNYIQLAQLEYALLARHLPAAPPPSRLAFLGSGPLPLSSLVLAARHLPAASFHNYDICADANR 179
AtNAS1     FPYYSNYLKLGKLEFDLLSQHSSHVPT--KIAFVGSGPMPLTSIVLAKFHLPNTTFHNFDIDSHANT 164
CLN        FPYFDNYIKLSLLEYNILTKNTTNIPK--KIAFIGSGPLPLTSLVLATKHLKTTCFHNYDIDVDANF 161
           ***: **: *  **  :* :          ::**:****:*::*:***  **  : * *:*    **

ZmNAS1     RARKLVRADKD-LNARMSFHTVDVANLTDELAKYDVVFLAALVGMAAEDKAKVVAHLGRHMADGAAL 230
ZmNAS2-1   RARKLVRADKD-LNARMSFHTVDVANLTDDLGKYDVVFLAAIVGMAAEDKAKVVAHLGRHMADGAAL 230
ZmNAS2-2   RARKLVRADEG-LRKQMFFHTADVANLTDELRKYDVVFLAALVGMAAEDKAKVVAHLGRHMVDGAAL 491
HvNAS1     RARKLFGATADGVGARMSFHTADVADLTQELGAYDVVFLAALVGMAAEEKAKVIAHLGAHMVEGASL 230
OsNAS1     RARRLFRGADEGLGARMAFHTADVATLTGELGAYDVVFLAALVGMAAEEKAGVIAHLGAHMADGAAL 231
ZmNAS3     RARRLVRADAA-LAARMAFRTSDVAHVTRELAAYDVVFLAALVGMAAEEKARVVEHLGRHMAPGAAL 262
OsNAS3     RASRLVRADRD-LSARMAFHTSDVAHVTTDLAAYDVVFLAALVGMAAEEKARMVEHLGKHMAPGAAL 246
AtNAS1     LASNLVSRDPD-LSKRMIFHTTDVLNATEALDQYDVVFLAALVGMDKESKVKAIEHLEKHMAPGAVL 230
CLN        MASALVAADPD-MSSRMTFHTADVMDVTCALKDYDVVFLAALVGMDKEDKVKVVDHLAKYMSPGATL 227
            *  *       :  ** *:* **   *  *  ************  * *   : **  :*  ** *

ZmNAS1     VVRSAHGARGFLYPIVDPEDIRRGGFDVLAVYHPDNEVINSVIIARKMDAHTKGLQNGHV---HARG 294
ZmNAS2-1   VVRSAHGARGFLYPIVDPEDIRRGGFDVLTVYHPDDEVINSVIIARKIDAHA---------------- 282
ZmNAS2-2   VVRSAHGARGFLYPIVDPEDIRRGGFDVLAVYHPDDEVINSVIVARKINAHVKGLQDGH---AHARG 555
HvNAS1     VVRSAR-PRGFLYPIVDPEDIRRGGFEVLAVHHPEGEVINSVIVARKAVEAQLSGPQNG--DAHARG 294
OsNAS1     VVRTAHGARGFLYPIVDPEDVRRGGFDVLAVCHPEDEVINSVIVARKVGAAAAAAAARRDELADSRG 298
ZmNAS3     VVRSAHGARGFLYPVVDPEEIRRGGFDVLAVHHPEGEVINSVIIARKPLPVVDEHAVAGVGHAHAHG 329
OsNAS3     VVRTAHGARGFLYPVVDPEEIRRGGFDVLAVHHPEGEVINSVIIARN-RPWPGPALEGGDAHAHGHG 313
AtNAS1     MLRRAHALRAFLYPIVDSSDLK--GFQLLTIYHPTDDVVNSVVIARKLGGPTTPGVNGTR------- 288
CLN        MLRSAHGARAFLYPVLDPRDLR--GFEVLSVYHPTDEVINSVIIARKLPVPSVPLLDGLG------A 286
           ::* *:  * ****::*  :::  **::*:: **  :*:***::**:

ZmNAS1     TV-PIVSPPCKCC-KMEANAL--QKREEMATTTELSI- 327
ZmNAS2-1   ------------------------------------- 
ZmNAS2-2   AV-PIVSPPCKCC-KMVANTL-HQKREEMATA------ 601
HvNAS1     AV-PLVSPPCNFSTKMEASAL--EKSEELTAKELAF-- 327
OsNAS1     VVLPVVGPPSTCC-KVEASAV--EKAEEFAANKELSV- 332
ZmNAS3     AV---LSRPCLCC-EMEARA--HQKMEEVAMEQLPS-- 359
OsNAS3     AV---VSRPCQRC-EMEARA--HQKMEDMSAMEKLPSS 345
AtNAS1     ---GCMFMPCN-CSKIHAIMNNRGKKNMIEEFSTIE-- 320
CLN        YVLPS----KCACAEIHA-FNPLNKMNLVEEFALEE-- 317
                          *     * :
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Figure 2. Comparison of the putative amino acid sequences of ZmNAS1 to ZmNAS3 with those of other NASs. HvNAS, barley NAS; OsNAS, rice NAS; AtNAS, Arabidopsis NAS; and CLN, tomato NAS. Asterisks indicate identical amino acid residues in all sequences. ZmNAS2-1 and ZmNAS2-2 signify the first and second NAS-like sequences in ZmNAS2, respectively. The terminal amino acid of ZmNAS2-1 (A) is followed by the first amino acid of ZmNAS2-2 (N) (see Fig. 3).

expressed sequence tag clone (GenBank accession no. Q619504) judging from its predicted molecular mass and pI value. Spots corresponding to ZmNAS1 and ZmNAS2 were larger than those of ZmNAS4 and ZmNAS5; therefore, we concluded that ZmNAS1 and ZmNAS2 are the most abundant known NAS isoforms in Fe-deficient maize roots.

NAS Activity of ZmNAS1, ZmNAS2, and ZmNAS3 Gene Products

To confirm the enzymatic function of their gene products, *ZmNAS* genes were fused to the maltose-binding protein (MBP) gene and the resulting fusion proteins were produced in *Escherichia coli*. MBP-

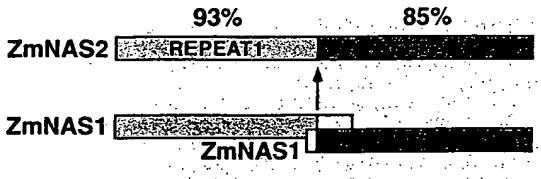

Figure 3. Schematic illustration of ZmNAS2 aligned with ZmNAS1. Repeats 1 and 2 indicate amino acids 1 to 281 and 282 to 601, respectively. The percentages of ZmNAS1 amino acids' identity to ZmNAS2 are indicated.

NASs were purified using amylose resin affinity columns, and 1 μg of each fusion protein was used for each enzyme assay. MBP-ZmNAS1 and MBP-ZmNAS3 displayed NAS activity (Fig. 6). In contrast, MBP-ZmNAS2 showed no NAS activity, even though ZmNAS2 has two NAS-like domains.

Localization of ZmNAS-Synthesized Green Fluorescent Protein (sGFP) Fusion Protein in Onion (*Allium cepa*) Epidermal Cells

The ZmNAS protein fused to the N terminus of sGFP was transiently expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter in onion epidermal cells. The green fluorescence of sGFP was viewed with a confocal microscopy. When sGFP alone was expressed (Fig. 7A), the protein was localized in the cytoplasm and nucleus. The fluorescence of ZmNAS1-sGFP and ZmNAS2-sGFP was observed as spots in the cytoplasm of onion epidermal cells (Fig. 7, B and C). On the other hand, the fluorescence of ZmNAS3-sGFP was distributed throughout the cytoplasm of onion epidermal cells (Fig. 7D).

DISCUSSION

Two Different Expression Patterns with Different Localization

The *ZmNAS3* gene was isolated by screening a genomic library using a heterologous probe. Subsequently, using *ZmNAS3* as a probe, two genes that encode NAS-like proteins, *ZmNAS1* and *ZmNAS2*, were isolated from a cDNA library prepared from Fe-deficient maize roots. The molecular masses of ZmNAS1 and ZmNAS3 were similar to previously characterized NAS isoforms (Fig. 2); however, ZmNAS2 had a higher molecular mass and contained two NAS-like sequence domains (Fig. 3). ZmNAS1 and ZmNAS3 showed NAS activity in vitro, but ZmNAS2 did not (Fig. 6).

Although ZmNAS2 did not exhibit NAS activity, the expression of *ZmNAS1* and *ZmNAS2* was coordinated, with both genes being up-regulated by Fe deficiency and suppressed by a resupply of Fe (Fig. 4). Transcripts of both genes were detected in Fe-deficient roots, but not in Fe-deficient leaves, not even in chlorotic leaves. This is similar to the case in barley, in which there is no *HvNAS* gene expression in leaves (Higuchi et al., 2001). Interestingly, transcripts of *OsNAS1* and *OsNAS2*, the expression of which is up-regulated by Fe deficiency, were detected not only in rice Fe-deficient roots, but also in chlorotic leaves (Higuchi et al., 2001). Therefore, although maize and rice are similarly susceptible to Fe deficiency, the expression patterns of *NAS* genes differ in response to Fe deficiency. In contrast to *ZmNAS1* and *ZmNAS2*, *ZmNAS3* was expressed under Fe-sufficient conditions, and its transcript level was decreased by Fe deficiency and was induced by an Fe resupply (Fig. 4). This is the first report of an *NAS* gene that is down-regulated by Fe deficiency in nongraminaceous or graminaceous plants. The decrease in *NAS* transcript levels in nongraminaceous plants in response to Fe deficiency was predicted based on the result that NAS enzyme activity is reduced in Fe-deficient roots of tobacco (*Nicotiana tabacum*; Higuchi et al., 1995). However, down-regulation of an *NAS* gene in graminaceous plants is unexpected

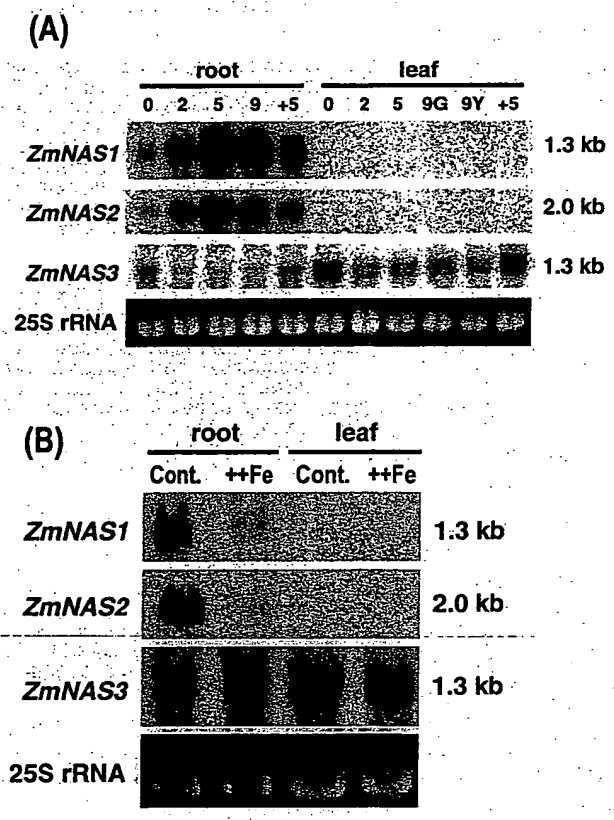

Figure 4. Northern-blot analysis of *ZmNAS* gene expression in maize during Fe deficiency and after resupply of Fe (A) and during Fe excess treatment (B). +5 indicates the number of days after Fe resupply. G and Y indicate green and yellow leaves of Fe-deficient plants, respectively. ++Fe indicates plants under Fe-excess conditions. A specific probe for each gene was used for hybridization. Equal loading of total RNAs was confirmed by ethidium bromide staining of 25S RNA.

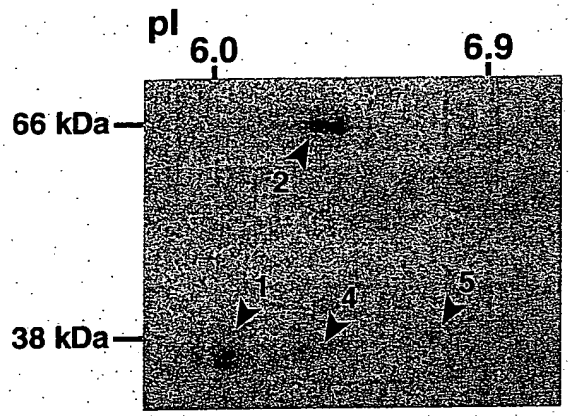

Figure 5. Western-blot analysis of NAS-like proteins in Fe-deficient maize roots. Figures indicate spots corresponding to putative *ZmNAS* gene products. 1, ZmNAS1; 2, ZmNAS2; 4, ZmNAS4; and 5, ZmNAS5.

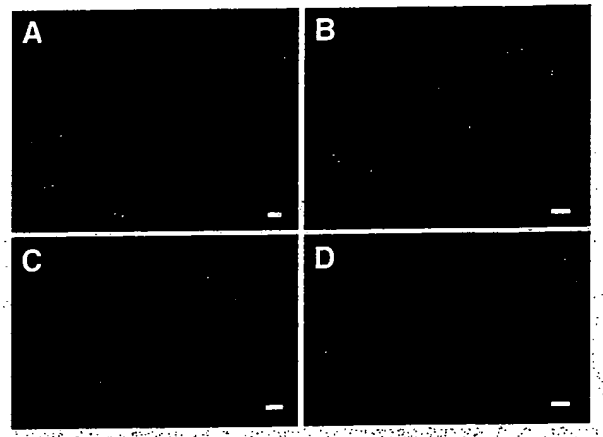

Figure 7. Subcellular localization of the transiently expressed ZmNAS-sGFP fusion protein in onion epidermal cells observed by confocal laser scanning microscopy. A, Onion epidermal cells expressing sGFP alone. B, Onion epidermal cells expressing ZmNAS1-sGFP fusion protein. C, Onion epidermal cells expressing ZmNAS2-sGFP fusion protein. D, Onion epidermal cells expressing ZmNAS3-sGFP fusion protein. Bars = 20 μm.

because the biosynthesis of phytosiderophores is greatly enhanced by Fe deficiency.

The localization of ZmNAS1-sGFP fusion protein and ZmNAS2-sGFP fusion protein was different from that of ZmNAS3-sGFP fusion protein. ZmNAS1-sGFP and ZmNAS2-sGFP were localized as small spots in the cytoplasm, whereas ZmNAS3-sGFP was distributed throughout the cytoplasm. PSORT (http://psort.nibb.ac.jp/) predictions were determined using full-length predicted protein sequences. All of the ZmNAS proteins were predicted to be localized at the membrane of the endoplasmic reticulum. Therefore, the pattern of ZmNAS1-sGFP and ZmNAS2-sGFP localization is interpreted as vesicles derived from the endoplasmic reticulum. The prediction of the ZmNAS3 localization did not match the result from the ZmNAS3-sGFP fusion, which indicated cytosolic localization. We have previously shown that "particular vesicles" in the Fe-deficient barley root cell is related to the secretion of MAs (Nishizawa and Mori, 1987). These data are consistent with the idea that NAS proteins induced by Fe deficiency in roots are localized at the membrane of vesicles derived from the endoplasmic reticulum, and that the vesicle is the place for the synthesis of MAs. On the other hand, ZmNAS3, localized throughout the cytoplasm, appears to synthesize NA itself, not as the precursor of MAs in the vesicles. ZmNAS3 has a longer amino-terminal region (Fig. 2) and this region was hydrophilic, which may affect the subcellular localization of ZmNAS3.

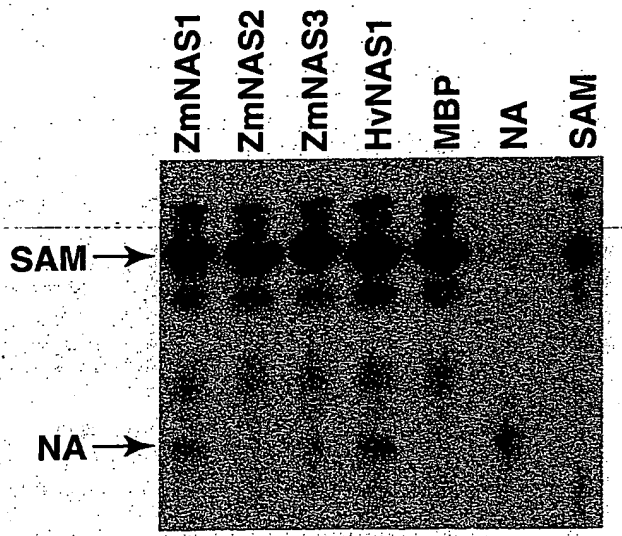

Figure 6. Thin-layer chromatography analysis of NAS activity in assay mixtures of MBP-NAS fusion proteins using 1 μg of each fusion protein or MBP for the enzyme assay. Lane SAM contains standard SAM; lane NA contains standard NA.

NA is essential not only for Strategy II plants, but also for Strategy I plants (nongraminaceous plants). The *chloronerva* mutant of tomato (*Lycopersicon esculentum*), which lacks NAS activity (Higuchi et al., 1996b), is not able to use Fe correctly (Becker et al., 1995). This mutant shows symptoms typical of Fe deficiency (Stephan and Grün, 1989), including intercostal chlorosis in young leaves, although it accumulates more Fe in all tissues than do wild-type plants (Scholz et al., 1985; Becker et al., 1992). Grafting the *chloronerva* mutant onto the wild type, or vice versa, restores the normal phenotype (Böhme and Scholz, 1960), and exogenous application of NA is also able to revert the phenotype (Budesinsky et al., 1980). These facts indicate that NA is necessary for Fe homeostasis in nongraminaceous plants. The expression of *ZmNAS3* under Fe-sufficient conditions implies that NA also plays an important role in Fe homeostasis in graminaceous plants. In support of this, Curie et al. (2001) recently isolated the *YS1* gene

from maize that encodes a MAs-Fe(III) complex transporter. Interestingly, Arabidopsis also possesses eight homologs to *YS1*, although it does not produce or use MAs because of the different strategy for Fe acquisition (Marschner et al., 1986; Guerinot and Yi, 1994; Briat and Lobréaux, 1997). These *YS1-like* genes have been proposed to encode NA-metal complex transporters (Walker, 2002). Rice also possesses at least 16 *YS1-like* genes, and some of their products may function as transporters of NA-metal complexes. These results imply that in nongraminaceous and graminaceous plants, NA is synthesized under Fe-sufficient conditions, and NA-Fe complexes are transported into and out of cells or subcellular organelles via Yellow Stripe-Like transporters. In contrast, NA synthesized in roots in response to Fe deficiency is used as a precursor in synthesis of MAs.

NA is also thought to play an important role in the detoxification of excess intracellular Fe (von Wirén et al., 1999). NA concentrations in tomato increase in response to Fe overload (Pich et al., 2001). Northern-blot analysis under Fe excess conditions revealed that the expression of *ZmNAS1* and *ZmNAS2* was repressed, and that of *ZmNAS3* was not affected by excess Fe.

The unrooted phylogenic tree (Fig. 8) suggests that

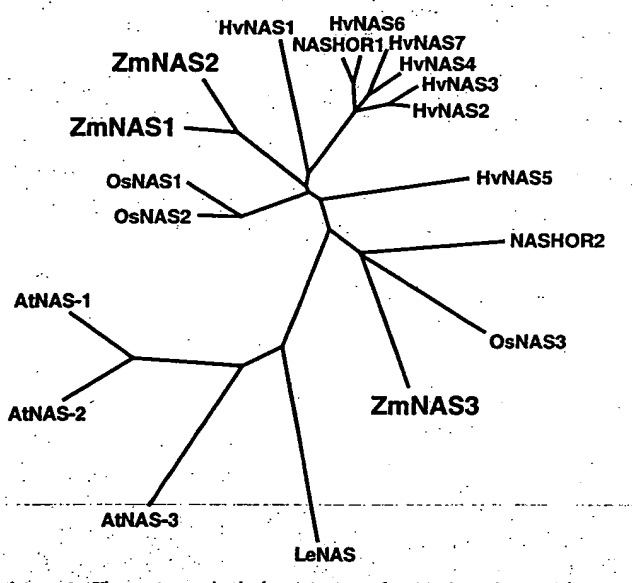

Figure 8. The unrooted phylogenic tree for NAS amino acid sequences for which open reading frames are confirmed. ZmNAS1 to 3 are from maize; HvNAS1 to 7 and NASHOR1 and 2 from barley; OsNAS1 to 3 from rice; AtNAS1 to 3 from Arabidopsis; and CLN from tomato. Calculations were performed using the CLUSTAL W neighbor-joining method and the tree was visualized with TreeView. Accession numbers of genes are: AB061270 (ZmNAS1), AB061271 (ZmNAS2), AB042551 (ZmNAS3), AB010086 (HvNAS1), AB011265 (HvNAS2), AB011264 (HvNAS3), AB011266 (HvNAS4), AB011268 (HvNAS5), AB011269 (HvNAS6), AB019525 (HvNAS7), AF136941 (NASHOR1), AF136942 (NASHOR2), AB021746 (OsNAS1), AB023818 (OsNAS2), AB023819 (OsNAS3), AB021934 (AtNAS1), AB021935 (AtNAS2), AB021936 (AtNAS3), and AJ242045 (CLN).

NASs in graminaceous plants are also classified into two groups as are ZmNAS proteins. OsNAS3 and NASHOR2 have a longer amino-terminal region and are closely related to ZmNAS3 in the unrooted phylogenic tree. In fact, *OsNAS3* was expressed in leaves under Fe-sufficient conditions and was suppressed by Fe deficiency, as is *ZmNAS3* (H. Inoue, personal communication). Presumably, *NASHOR2* has similar characteristics to *ZmNAS3* and *OsNAS3* and may show a similar expression pattern in barley. Seven of nine *HvNAS* genes were isolated from an Fe-deficient root cDNA library (Higuchi et al., 1999a), and *NASHOR2* was isolated from a seed-specific cDNA library (Herbik et al., 1999). These facts also support the idea that *NASHOR2* is expressed in Fe-sufficient barley leaves and is suppressed by Fe deficiency.

Inactive ZmNAS2 in Spite of the Duplicated Structure

HvNAS1 showed NAS activity in vitro as a MBP fusion (MBP-HvNAS1; Fig. 6). Three MBP fusions of Arabidopsis NAS proteins (AtNAS1, AtNAS2, and AtNAS3) were also active in vitro (Suzuki et al., 1999). These results suggest that other MBP-NAS fusions should also exhibit NAS activity when produced in *E. coli*, supported by the finding that MBP-ZmNAS1 and MBP-ZmNAS3 were as active in vitro as MBP-HvNAS1. In contrast, MBP-ZmNAS2 was completely inactive under the same conditions (Fig. 6), despite the fact that *ZmNAS2* contains partially duplicated *NAS* sequences. Båga et al. (2000) described a starch-branching enzyme I in wheat (*Triticum aestivum*) that contains partially repeated starch-branching enzyme I sequences and exhibits branching-enzyme activity. NAS catalyzes the complicated reaction in which three SAM molecules are conjugated into one NA molecule in a single step (Fig. 1B; Higuchi et al., 1999b). Therefore, it is conceivable that highly accurate protein conformation is required for adequate NAS activity. The *chloronerva* mutant harbors a single base mutation resulting in the change of one amino acid that is otherwise conserved among all other NAS family members. This mutation in the NAS protein strongly diminishes NAS activity in this mutant (Ling et al., 1999). Although ZmNAS2 possesses this conserved region in both of the NAS repeats, it has no NAS activity in vitro as a MBP fusion. The first NAS repeat in ZmNAS2 has one amino acid difference, L205I, in another highly conserved region, and this may prohibit NAS activity by this protein.

As described earlier, NAS is responsible for tolerance to low Fe availability in graminaceous plants. Many genes encoding NAS proteins have been isolated from graminaceous plants: nine from barley (Herbik et al., 1999; Higuchi et al., 1999b), three from rice (Higuchi et al., 2001), and three from maize, as reported in this study. In addition, we have identified many NAS-like expressed sequence tag clones

from other graminaceous plants in public databases, including those for wheat, *Sorghum bicolor*, and winter rye. These data suggest that graminaceous plants, including maize, have increased the number of *NAS* genes, thus enhancing their tolerance of Fe deficiency. However, in the case of maize, it is possible that *ZmNAS2* remains a fused gene because the two genes were not able to separate completely. Higuchi et al. (1999b) reported the presence of a NAS-like protein similar to ZmNAS2 in molecular mass and pI value in Fe-deficient *S. bicolor* roots. Such a large NAS-like protein has been detected only in maize and *S. bicolor* (subfamily Panicoideae). The failed *NAS* duplication event that produced *ZmNAS2* thus probably occurred before maize and *S. bicolor* diverged.

It is possible that ZmNAS2 has some biological function. It is conceivable that a proteolytic cleavage takes place, producing an active enzyme when needed. We detected an uncloned NAS isoform (ZmNAS5) in Fe-deficient maize roots, possibly arising from the cleavage of ZmNAS2. In the absence of this cleavage, it is possible that the long polypeptide manages to titrate the substrate and lead to some kind of regulation.

A recent study showed that transgenic rice containing *NAAT* genes from barley is more tolerant than nontransgenic individuals to low Fe availability in calcareous soil (Takahashi et al., 2001). By introducing *NAS* genes such as HvNAS1, along with other genes involved in MAs synthesis (Fig. 1), we can expect to produce a transgenic maize that tolerates Fe deficiency by increasing the amount of secreted MAs. Increasing the productivity of maize in calcareous soils will help meet the increased demand for food that is expected owing to rapid population increases, increased biomass production for fuels, and reduce CO_2 concentration by covering calcareous soils.

MATERIALS AND METHODS

Plant and Culture Conditions

Seeds of maize (*Zea mays* cv Alice) were germinated on paper towels soaked with distilled water in a tray covered with a sheet of aluminum foil for 5 d at 27°C. After germination, plants were transferred to a polythene net floating on standard culture solution (pH 5.5) and were placed in a growth chamber under conditions of 30°C/16 h of light and 25°C/8 h of dark. The standard culture solution consisted of 2×10^{-3} M $Ca(NO_3)_2$, 7×10^{-4} M K_2SO_4, 1×10^{-4} M KCl, 1×10^{-4} M KH_2PO_4, 5×10^{-4} M $MgSO_4$, 1×10^{-5} M H_3BO_3, 5×10^{-7} M $MnSO_4$, 5×10^{-7} M $ZnSO_4$, 2×10^{-8} M $CuSO_4$, 1×10^{-8} M $(NH_4)_6Mo_7O_{24}$, and 1.5×10^{-4} M Fe-EDTA. After 4 d, plants were transplanted into 20-liter plastic boxes and were cultured for 5 d in standard culture solution with adequate amounts of Fe. Plants were then kept in standard culture solution without Fe for 9 d. Finally, Fe was added to the culture solution in the form of 6.0×10^{-4} M Fe-EDTA. For preparing the Fe-excess plants, 1.0×10^{-3} M Fe-EDTA was added to the culture solution and plants were harvested after 10 d. Culture solutions were prepared with distilled water, adjusted to pH 5.5 every day using 1.0 M HCl, and renewed every 5 d. Roots and leaves were harvested at various stages, frozen in liquid nitrogen, and stored at −80°C until use.

Cloning of *ZmNAS3* from a Maize Genomic Library

To isolate an *NAS* homolog, a genomic library (Corn Genomic Library, variety B73; CLONTECH) was screened using plaque hybridization techniques and a probe consisting of the open reading frame of the rice (*Oryza sativa*) *NAS* gene (*OsNAS1*; accession no. AB021746). The probe was labeled with [α-^{32}P]dATP using a Random Primer Labeling kit (version 2; TaKaRa, Kusatsu, Japan) and was purified using a microcolumn (ProbeQuant G-50; Pharmacia, Uppsala). Of approximately 400,000 plaques, one positive clone was plaque purified and its insert was subcloned into the pBluescriptII (SK-) vector. This clone was designated *ZmNAS3* (accession no. AB042551).

Screening a Maize cDNA Library

Total RNA was extracted from Fe-deficient maize roots using the SDS-phenol method. A cDNA library was constructed using the cloning vector pSPORT1 (Invitrogen, Carlsbad, CA) as described previously (Higuchi et al., 2001). Approximately 400,000 colonies of the cDNA library were screened for other *NAS* clones using colony hybridization with a probe consisting of a 0.7-kbp *PstI*/*SalI* fragment from *ZmNAS3*. Isolated cDNA clones were sequenced using a Thermo Sequenase Cycle Sequencing kit (Shimadzu, Kyoto) and a DNA sequencer (DSQ-2000L; Shimadzu).

Northern-Blot Analysis

Northern-blot analyses were conducted using specific probes for *ZmNAS1*, *ZmNAS2*, and *ZmNAS3* labeled with [32P-]dATP. Specific primers for each gene were designed as follows and were used for preparing specific probes: *ZmNAS1*, 5′-GAGGAGATGGCGACCACGACAG-3′ and 5′-GAAGTGCATGAGAAATTCAGCA-3′; *ZmNAS2*, 5′-ATCGACGCCCATGCAAACAC-3′ and 5′-ATCCTCTGCGCGTCCGTGCC-3′; and *ZmNAS3*, 5′-GCCATGGCCGTCATGGGCAA-3′ and 5′-ATCTTGGCACCAGCGCCGACT-3′. Total RNA was extracted from maize roots and leaves using the SDS-phenol method. Ten micrograms of total RNA was separated, blotted, and hybridized with the probes at 42°C as described previously (Higuchi et al., 1999b).

Preparation of Polyclonal Antibodies to NAS (Higuchi et al., 1999a)

Two mice were immunized with a total of 100 μg of NAS peptides prepared from Fe-deficient barley (*Hordeum vulgare*) roots. For the first injection, the immunogen was emulsified in complete Freund's adjuvant. For the second and subsequent infections, incomplete Freund's adjuvant was used. After the fourth induction, whole blood was collected and the antiserum was stored at −80°C until use.

Western-Blot Analysis

Proteins were extracted from Fe-deficient maize roots, separated using two-dimensional PAGE, and blotted as described previously (Higuchi et al., 1999a). Western-blot analysis was performed using polyclonal NAS antibodies prepared by Higuchi et al. (1999a), along with a secondary antibody, goat anti-mouse IgG (H+L) conjugate and horseradish peroxidase (Wako, Osaka). The blot was stained with diaminobenzidine.

Expression of Recombinant ZmNAS Proteins in *Escherichia coli*

To subclone *ZmNAS1* into pMAL-c2 (New England Biolabs, Beverly, MA), an annealed oligomer (5′-CCATGCGGAATTCCG-3′) was inserted into the *NcoI* site (including the first ATG) of *ZmNAS1* cloned in pSPORT. This *ZmNAS1* plasmid was then digested with *EcoRI* and *HindIII*, and the excised fragment containing the *ZmNAS1* coding sequence was subcloned into pMAL-c2. To subclone *ZmNAS2* into pMAL-c2, an *EcoRI* site was introduced close to the first ATG using PCR mutagenesis, using two primers: 5′-GAGACTCTGAATTCGCCATGGAGGCCCAGAACGTGGA-3′ and 5′-GAGACTCTAAGCTTCATATGAGTTCCATGCATCAGATGGACA-3′. The amplified fragments were digested with *EcoRI* and *HindIII*, and the excised fragment was cloned into pBluescriptII (SK-). This clone was des-

ignated *ZmNAS2B*. We exchanged the region between *Eco*RI and *Sma*I of *ZmNAS2* in pSPORT with the *Eco*RI/*Sma*I *ZmNAS2B* fragment. The resulting *ZmNAS2* construct was digested with *Eco*RI and *Hin*dIII, and the excised fragment containing the *ZmNAS2* coding sequence was subcloned into pMAL-c2. To subclone *ZmNAS3* into pMAL-c2, an *Eco*RI site was introduced close to the first ATG using PCR mutagenesis, using two primers: 5′-GAGACTCTGAATTCCATATGGCCGTCATGGGCAAGGAG-3′and 5′-GAGACTCTGGATCCCACTTAATACAATCACGGTGAC-3′. The amplified fragments were digested with *Eco*RI and *Bam*HI, and the excised fragment was cloned into pBluescriptII (SK-). The resulting *ZmNAS3* construct was digested with *Eco*RI and *Xba*I, and the excised fragment containing the *ZmNAS3* coding sequence was subcloned into pMAL-c2. These pMAL-c2 plasmids, containing the coding sequences of *ZmNAS*, were introduced into *E. coli* XL1-Blue, which was induced to produce the recombinant fusion proteins. These proteins were purified as described previously (Higuchi et al., 1999b).

Assay of NAS Activity

NAS enzyme activity was assayed using the cell-free system modified by Higuchi et al. (1994) and Suzuki et al. (1999). One microgram of each fusion protein was added to the reaction buffer [50 mM Tris-HCl, 1 mM EDTA, 3 mM dithiothreitol, 10 μM (*p*-amidinophenyl) methanesulfonyl fluoride (*p*-APSMF), and 10 μM trans-epoxysuccinyl-L-leucylamido(4-guanidino) butane (E-64), pH 8.7] and was concentrated by ultrafiltration using a filter (Ultrafree C3LGC NMWL10000; Millipore, Bedford, MA). [^{14}C]SAM was added to the concentrated enzyme solution to a final concentration of 20 μM. After a 20-min incubation at 25°C, 5 M HCl was added to a final concentration of 0.2 M to stop the enzyme reaction. [^{14}C]NA was separated on thin-layer chromatography LK6 plates (Whatman, Clifton, NJ), which were developed with a phenol:n-butanol:formate:water solution (12:3:2:3, v/v). [^{14}C]NA was then detected using an image analyzer (BAS2000; Fuji Film, Tokyo). Standard [^{14}C] NA was synthesized from [^{14}C]SAM using crude extracts of NAS prepared from Fe-deficient barley roots.

Construction of Plasmid ZmNAS-sGFP

Plasmid pUC18 containing the construct, CaMV 35S promoter-*sGFP* (S65T)-NOS3′, was kindly provided by Dr. Yasuo Niwa (University of Shizuoka, Shizuoka, Japan). The construct had *Sal*I and *Nco*I sites on the 3′ side of the CaMV 35S promoter. The *Nco*I site "CCATGG" included the initiation codon for *sGFP*. An annealed oligomer (5′-TCGAGGGCCC-3′) was inserted into the *Sal*I site of CaMV35S-sGFP (S65T)-NOS3′ to produce *Apa*I site "GGGCCC." This modified plasmid was designated CaMV35S-*Apa*I-sGFP (S65T)-NOS3′. The open reading frame of *ZmNAS1* was amplified using two primers: 5′-GAGACTCTGGGCCCATGGAGGCCCAGAACGTGGA-3′ and 5′-GAGACTCTGAATTCATGAAGATGGACAGCTCTGTCGTGG-3′. The amplified fragments were digested with *Apa*I and *Eco*RI, and the excised fragment was cloned into pBluescriptII (SK-). The resulting *ZmNAS1* construct was digested with *Apa*I and *Bsp*HI, and the excised fragment containing the *ZmNAS1* coding sequence was subcloned into CaMV35S-*Apa*I-sGFP (S65T)-NOS3′ digested with *Apa*I and *Nco*I. The open reading frame of *ZmNAS2* was amplified using two primers: 5′-GAGACTCTGGGCCCATGGAGGCCCAGAACGTGG A-3′ and 5′-GAGACTCTGAATTCATGAACGCCGTTGCCATCTCCTCCCT-3′. The amplified fragments were subcloned into CaMV35S-*Apa*I-sGFP (S65T)-NOS3′ in the same way.

Two annealed oligomers, 5′-TCGAACCATGGAGCAGCTGCCGTCCGG-3′ and 5′-CATGCCGGACGGCAGCTGCTCCATGGT-3′, which encoded five N-terminal amino acid residues of ZmNAS3, were inserted into the *Sal*I/*Nco*I site of CaMV35S-sGFP(S65T)-NOS3′. The *Nco*I fragment excised from *ZmNAS3* was inserted in to the *Nco*I site of the resulting plasmid.

Transformation and Microscopic Observation of Onion (*Allium cepa*) Epidermal Cells

Transformation of onion epidermal cells was carried out by the Biolistic PDS-1000/He Particle Delivery System (Bio-Rad, Hercules, CA). White onion bulbs were purchased locally and were stored in the dark until they were used. Inner epidermal layers were peeled and placed inside up on a Murashige and Skoog plate solidified with 2% (w/v) Gellan Gum. Gold particles with a diameter of 1.0 μm were coated with each plasmid DNA containing the different constructs and were prepared for bombardment according to the manufacturer's protocol. Plated onion epidermal layers were placed under the stopping screen at a distance of 8 cm and were bombarded in a vacuum of 28 inches of mercury using a helium pressure of 1,350 psi to accelerate the macrocarrier. Bombarded cells were kept in the dark at 28°C for 20 h. Bombarded onion cells were mounted on a slide glass, and fluorescence viewed with a laser-scanning confocal microscope (LSM510; Karl Zeiss, Jena, Germany) equipped with an argon laser and a GFP filter set.

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Directed Evolution of Yeast Ferric Reductase to Produce Plants with Tolerance to Iron Deficiency in Alkaline Soils

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One of the most serious problems in agriculture worldwide is low iron (Fe) availability, due to high soil pH. About 30% of arable land is too alkaline for optimal crop production. Non-graminaceous and dicot plants, which use a reduction strategy to uptake Fe, suffer from Fe deficiency under these conditions, because the ferric chelate reductase in the root plasma membrane functions inefficiently at high pH. The *refre1* (reconstructed yeast ferric reductase) gene was subjected to random mutagenesis to obtain variants with high activity under high pH conditions. A mutant library was screened using a yeast in vivo assay system, and screens at pH 8.0 and 8.5 produced 10 candidates. In vivo ferric reductase activity was analyzed quantitatively. Yeast cells carrying the variant with the highest ferric reductase activity showed 6.0, 8.7, and 38 times greater activity at pH 8.0, 8.5, and 9.0, respectively, than did cells containing the original *refre1* gene. An amino acid substitution at position 312 was common to most of the high-activity variants. This substitution is believed to play an important role in the increased reductase activity at high pH. Interestingly, this mutation is near a heme-coordinating histidine codon, and the corresponding residue is probably located in the intramembranous region close to the cytoplasm. The variant gene with the highest reductase activity was introduced into tobacco, and transgenic tobacco carrying the gene showed enhanced tolerance to low Fe availability. This result should be useful in the engineering of non-graminaceous and dicot plants tolerant to Fe deficiency in alkaline soils.

Key Words: **alkaline soil, directed evolution, ferric reductase, iron deficiency, *Nicotiana tabacum*.**

Fe is an essential element for almost all living organisms because of its critical role in processes such as DNA synthesis, respiration, and photosynthesis. Although Fe is abundant in soils, its biological availability is low because it primarily forms highly insoluble ferric compounds at neutral or basic pH. Fe deficiency is a worldwide agricultural problem, since calcareous soils cover about 30% of the earth's land area. Depending on the species, plants take up Fe as either ferric or ferrous ions (Mori 1988). To increase Fe availability in the rhizosphere under Fe-deficient conditions, dicots and non-graminaceous monocots increase their ferric reduction capacity at the root surface, enhance proton excretion in the rhizosphere, and release reductants and chelators (Römheld and Marschner 1983). Reduction by ferric chelate reductase is thought to be the rate-limiting step in Fe uptake (Grusak et al. 1990). The expression of the gene responsible for ferric reduction in *Arabidopsis thaliana*, *FRO2* (Robinson et al. 1999) involves post-transcriptional regulation, as shown for IRT1 (Connolly et al. 2002, 2003). Overexpression of the *FRO2* gene leads to improved growth in low-Fe conditions at pH 6.0. Previously, we reconstructed the yeast ferric reductase gene, *FRE1*, for functional expression in plants, designating the gene *refre1* (reconstructed *FRE1*) (Oki et al. 1999). However, in calcareous soils, the reduction

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steps catalyzed by both FRO2 and FRE1 are inhibited by the high pH of the soil. To overcome this problem, we improved the enzymatic properties of the ferric reductase encoded by the *refre1* gene using PCR-mediated random mutagenesis.

Random mutagenesis is a powerful tool for obtaining enzymes with specific properties (Arnold 1998). As most of the mutations are deleterious or neutral, large numbers of variants must be screened to isolate those with the desired characteristics, and a convenient assay system facilitates the process. Although the *refre1* gene uses *Nicotiana tabacum* codons, the gene is expressed well in yeast cells. The *FRE1* gene was originally identified from a yeast mutant lacking ferric reductase activity (Dancis et al. 1992), and the same process was used to screen for *refre1* variants.

MATERIALS AND METHODS

Yeast strains, media, and expression vector. *Saccharomyces cerevisiae* strain CM3260 (MATa *trp1-63 leu2-3, 112 gen4-101 his3-609 ura3-52*) was used. Yeast cells were grown in YPD medium or a synthetic medium (SD), including the necessary supplements. The plasmid vector pHO5, which is derived from Yeplac181 (Gietz and Sugino 1988) and contains the ADH promoter, ADH terminator (derived from pVT-100U [Vernet et al. 1987]), and the 2-μm *ori*, was used as the yeast expression vector.

Random mutagenesis. Error-prone PCR of the *refre1* gene was carried out in a 50-μL reaction mix containing 1.5 ng of plasmid DNA (pT7-blue(R) vector carrying the *refre1* gene), Perkin-Elmer PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w / v) gelatin, pH 8.3), 0.5 mM $MnSO_4$, 0.2 mM of each dNTP, 10 pmol of each primer, and 1 unit of AmpliTaq Gold™ (Perkin-Elmer). The PCR thermocycle profile used was 94°C for 9 min; 40 cycles of 94°C for 40 s, 55°C for 30 s, and 72°C for 1 min, with a final extension step of 72°C for 10 min. The primers used for gene amplification were as follows: forward, 5′-CGCCAGGGTTTTCCCAGTCACGAC-3′, and reverse, 5′-GAGCGGATAACAATTTCACACAGG-3′.

Library construction. Mutagenized *refre1* genes were fused into the yeast expression vector pHO5 between the *Hin*dIII and *Not*I sites. The plasmids were amplified once in the *Escherichia coli* strain XL-1 Blue on solid LB medium, followed by extraction of the plasmids using the alkaline-SDS method. Yeast cells were transformed with the constructed library using standard procedures (Gietz et al. 1995).

Screening of mutant colonies. Replicas were lifted from plates using 0.45-μm Biodyne A nylon membranes (Pall). The inverted replicas were incubated for 5 min at room temperature on Whatman 3MM filter paper soaked in assay buffer (50 mM sodium citrate, 50 mM HEPES, 0.2 mM $CaSO_4$). The membranes were then transferred to a second filter paper soaked in assay buffer plus 0.5 mM $FeCl_3$ and 0.5 mM bathophenanthroline disulfonic acid (BPDS), and incubated for 30 min. The membranes were dried and compared with the original plate to identify colonies with increased reductase activity (Fig. 1).

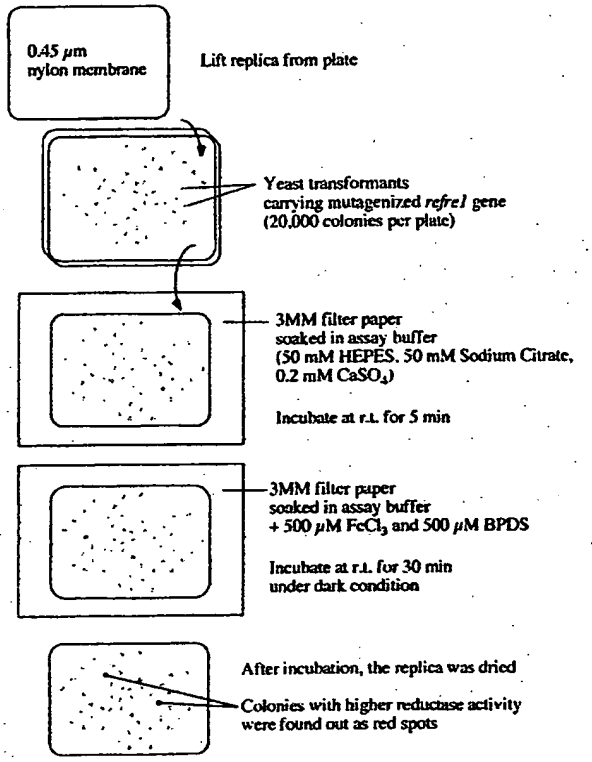


Fig. 1. Screening method. The screening system used in this study was the same method used to identify a yeast mutant lacking ferric reductase activity (Dancis et al. 1990), with the minor modifications of growing the transformed yeast cells in Fe-sufficient conditions and adjusting the pH of the assay buffer to 8.0 or 8.5.

Quantitative analysis. Yeast cells were grown to saturation in liquid SD-Leu medium, resuspended to a concentration of 1×10^6 cells per mL in liquid SD-Leu, and grown for 8 to 10 h. The cells were collected by centrifugation and incubated at 30°C in each assay buffer. After 30 min, BPDS and $FeCl_3$ were added to final concentrations of 0.5 mM and the incubation was continued for an additional 5–15 min. The cells were then removed by centrifugation and the optical density of the supernatant was measured at 520 nm. The amount of Fe^{2+} produced was estimated from a calibration curve constructed using solutions of known Fe^{2+} concentration.

Transformation of tobacco and testing for tolerance to Fe deficiency on calcareous soil. The coding region of variant 372 was used to replace the β-glucuronidase gene in pBI121 (Clontech). The transformation method and regeneration procedure used were as previously described (Oki et al. 1999), except the metal concentration of the regeneration medium was half the concentration normally used. T_1 plants were cultivated in pots filled with alkaline soil and set in a tray watered with a modified MS culture solution containing 10% of the normal concentration of the major elements and no added Fe, at a pH of 8.0. Once per week, the plant heights were measured and the degree of chlorosis of the youngest fully expanded leaf was determined using a SPAD-502 chlorophyll meter (Minolta Co., Tokyo, Japan). At 35 d after transfer to alkaline soils, the ferric reductase activities of the plants were measured by washing excised roots in 0.2 mM $CaSO_4$ for 5 min and incubating the roots in 150 mL of assay buffer (0.2 mM $CaSO_4$, 5.0 mM HEPES pH 8.0, 0.1 mM Fe(III) citrate, 0.2 mM BPDS) at 25°C for 30 min. The optical density of the solution at 535 nm was then measured. The amount of Fe^{2+} produced was estimated from a calibration curve constructed using solutions of known Fe^{2+} concentration. Plants were cultured in pots filled with an alkaline soil (pH 8.5) obtained from the Toyama Prefecture of Japan and grown in a greenhouse under natural light conditions (Takahashi et al. 2001).

RESULTS

Error-prone PCR was used to introduce random mutations into the *refre1* gene, which encodes a protein of 686 amino acids. The mutation rate was controlled to generate an average of five base substitutions per gene. Using a yeast in vivo assay system, the mutant library was screened for variants with greater ferric reductase activity than the original gene at high pH. Although the native yeast ferric reductase genes (FRE1 and FRE2) were not disrupted, the effects of these genes were negligible because the cells were grown in medium containing sufficient Fe. The screening strategy used is outlined in Fig. 1. Approximately 400,000 clones (20,000 clones per plate) were screened at pH 8.0 or pH 8.5. Positive clones isolated during the first screening were subjected to a second screening (100–200 colonies per plate). Plasmids were extracted from clones that were positive in the second screening and retransformed into yeast cells. Quantitative enzyme activity analysis was performed with these transformed cells to verify the initial results. The variant with highest activity, 372, showed 6.0, 8.7, and 38 times greater activity than the original refre1 protein at pH 8.0, 8.5, and pH 9.0, respectively

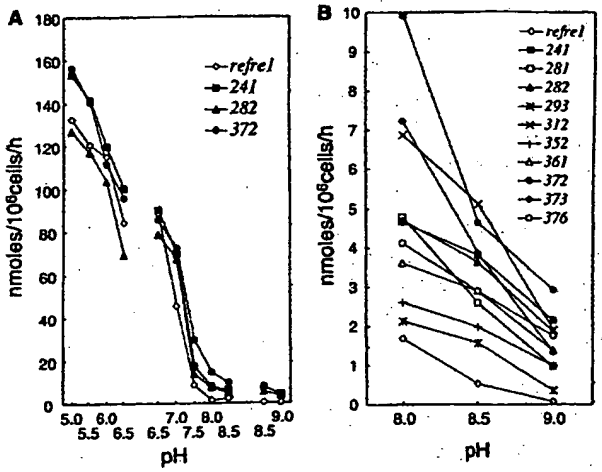


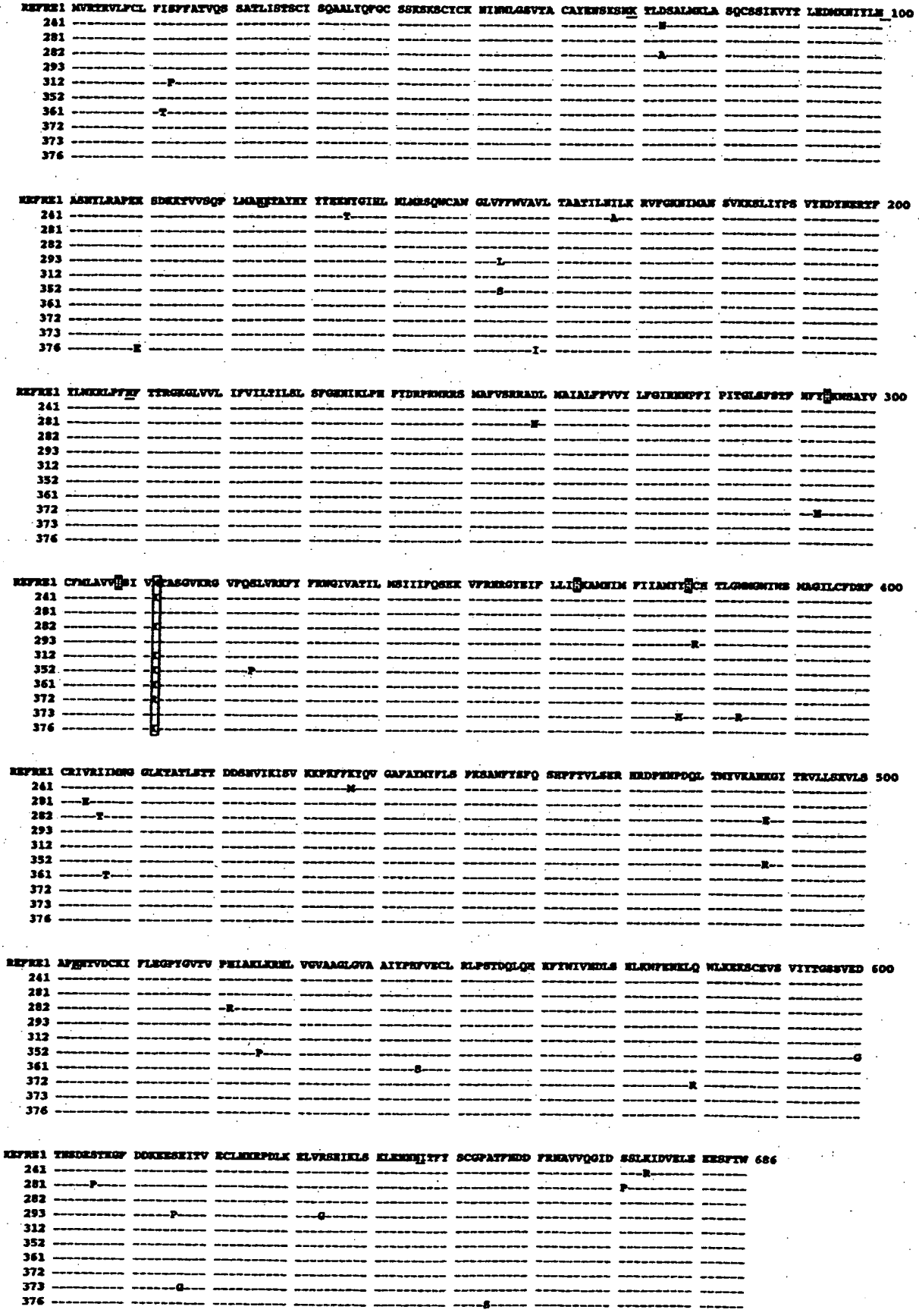
Fig. 2. Reductase activity of the variants. The assay buffers used for each experiment were: (A) 50 mM HEPES (pH 8.0 and pH 8.5), 50 mM TAPS (pH 9.0), 50 mM sodium citrate, and 0.2 mM $CaSO_4$, and (B) 50 mM MES (pH 5.0 to 6.5), 50 mM HEPES (pH 6.5 to 8.5), 50 mM TAPS (pH 8.5 and 9.0), 50 mM sodium citrate, and 5% glucose.

(Fig. 2A). This variant showed increased activity at all pH levels examined, but the activity was only 1.2 times greater than normal at pH 5.0 (Fig. 2B).

Although the error-prone PCR conditions used were designed to generate an average of 5 base substitutions per gene, up to 13 base substitutions were observed in the variant genes. The number of amino acid substitutions ranged from 2 to 6 (Fig. 3). Seven of the 10 variants had similar mutations at amino acid position 312, which is located near a heme-binding histidine residue (H308) (Figs. 3, 4). These mutations resulted in the substitution of a methionine codon with those for lysine or arginine. Variant 312 contains only two mutations: the mutation noted above, and another located in the first 22 amino acids, which conform to the von Heijne consensus for the leader peptide of a membrane or secreted protein (von Heijne 1983).

The gene for variant 372, which showed the greatest reductase activity of all of the variants, was introduced into tobacco using the *Agrobacterium* method, in conjunction with the cauliflower mosaic virus 35S promoter (Oki et al. 1999). Regenerated plants were obtained only

Fig. 3. Amino acid sequences of the variants. The original REFRE1, the amino acid sequence of which is identical to that of the yeast ferric reductase FRE1, is shown with the amino acid substitutions found in the variants. The boxed amino acid substitutions were common to most of the variants. The four histidine residues coordinating the two intermembranous heme groups are shaded in black, and potential sites for N-linked glycosylation are underlined.



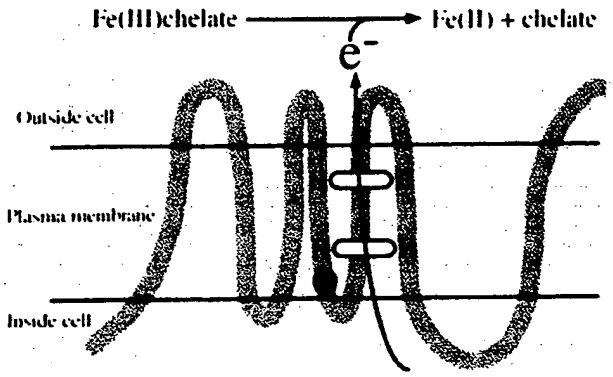


Fig. 4. Hypothetical structure of REFRE1 in the plasma membrane. The four histidine residues (H) coordinating the two intramembranous heme groups (white bars) (Finegold et al. 1996) and the critical point (M312, closed circle) are indicated.

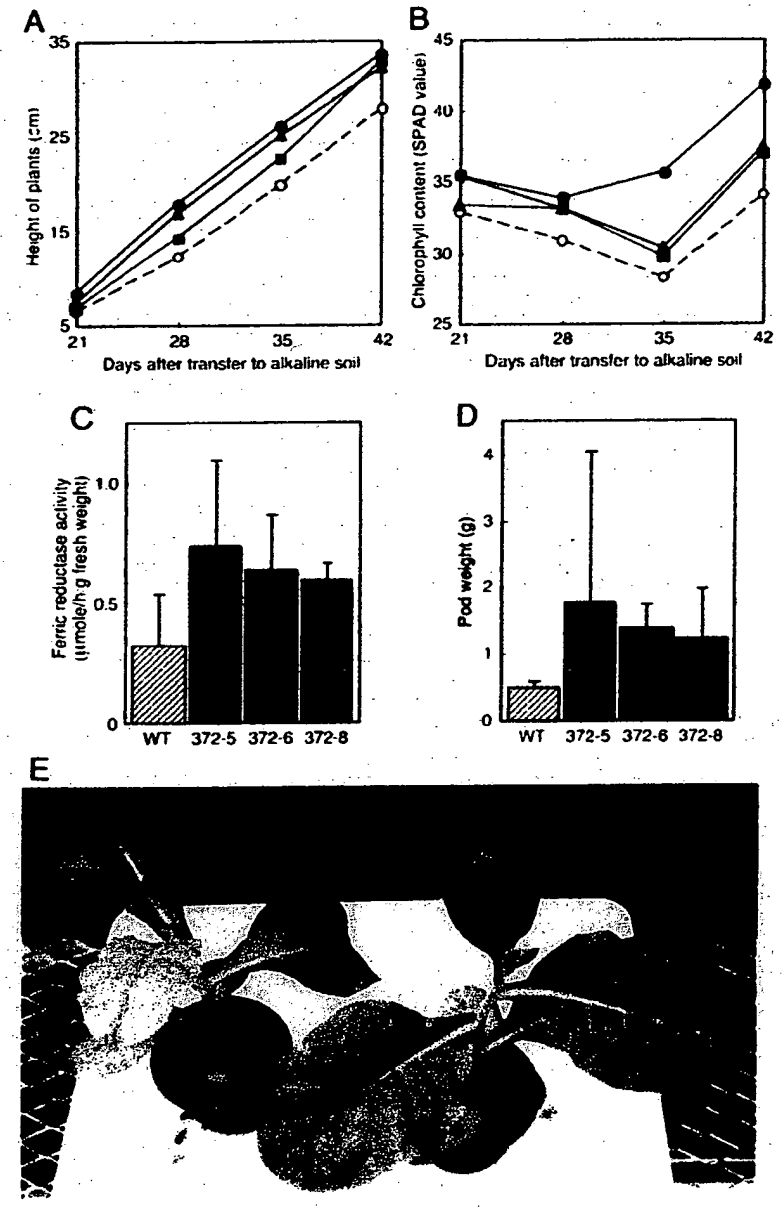


Fig. 5. Transgenic tobacco harboring the *refre1* (372) gene is tolerant to low Fe availability in alkaline soils. (A) Heights of the control wild-type plant (broken line) and three transgenic plants (solid lines) 21–42 d after transplantation into alkaline soil (pH 8.5). (B) Chlorophyll content (SPAD value) of the control wild-type plant (broken line) and three transgenic plants (solid lines). (C) Ferric reductase activity of roots of the control wild-type and transgenic plants after cultivation for 35 d in alkaline soils at pH 8.0, $n = 3$. (D) Final seed pod yield of the plants, $n = 3$. (E) Transgenic tobacco plants (right) and a control wild-type plant (left) grown in alkaline soils.

when the minor metal nutrient concentrations were decreased to half those of the normal MS medium; it is possible that transformed callus did not regenerate, due to the high reductase activity of the cells. Southern blot analysis showed that the transgene was efficiently integrated into the tobacco genome (not shown). Transgenic plants were cultured in an alkaline soil (pH 8.5) to examine the tolerance of the transformants to low Fe availability. Under these conditions, the transformants were tolerant of the low availability of Fe and appeared healthier than the control plants (Fig. 5A, E). The transformants had higher chlorophyll contents, higher reductase activity (Fig. 5B, C), and seed pod yields more than double that of the control (Fig. 5D).

DISCUSSION

A screen for *refre1* variants with increased ferric reductase activity under high pH conditions produced 10 variants. Sequencing of the variant genes revealed that the mutation of the codon M312 to a K or R codon is crucial for the increased activity at high pH. The FRE1 protein has seven potential transmembrane domains (Dancis et al. 1992) and is localized at the plasma membrane. The protein contains a bis-heme motif, and the heme groups that mediate electron transfer from cytosolic NAD(P)H to extracellular ferric chelates are integrated into the protein through four histidine residues, or two residues per heme (Finegold et al. 1996). The site of the above mutation is near a histidine residue, and a change near a heme-binding residue may improve the efficiency of heme loading or electron transport (Fig. 4). Except for the mutation at M312, the 10 variants had no common mutations. It is unknown whether electron transfer to extracellular ferric chelates occurs directly or indirectly. In this study, no common mutations were observed in regions that are predicted to be located extracellularly, where ferric chelates might be reduced. One explanation is that the area surrounding the site of the enzyme-substrate interaction already has an optimal sequence, but this is unlikely given the imperfections in enzyme evolution observed in nature, including those in the yeast FRE1. A more likely explanation is that there is indirect electron transport to ferric chelates or electron transport to ferric chelates without an interaction between the enzymes and substrates. It has been suggested that superoxide radicals are involved in the reduction of ferric chelates (Cakmak et al. 1987). This small intermediate can explain the large variety of ferric chelate substrates that exists (Shatwell et al. 1996). In the present study, no mutation was observed at the six potential sites for the addition of N-linked sugars (Dancis et al. 1992). This suggests that these sites are important for the activity of the enzyme, which could easily be examined using site-directed mutagenesis.

In graminaceous plants, the introduction of the barley nicotianamine aminotransferase gene into rice enhanced the secretion of phytosiderophores and the tolerance to low Fe availability in alkaline soils (Takahashi et al. 2001). The genes for two important components for Fe uptake from soil, the Fe^{2+} transporter IRT1 and the ferric reductase FRO2, have been isolated from non-graminaceous and dicot plants (Eide et al. 1996; Robinson et al. 1999; Vert et al. 2001; Waters et al. 2002). Investigation of the rate of Fe^{3+} reduction and ^{59}Fe uptake in Fe-deficient peanut (*Arachis hypogaea* L.) under unbuffered and buffered (pH 8.5 with 10 mM HCO_3^- or 10 mM HEPES) conditions showed that increasing the ferric reductase activity 2–3 times increased the Fe uptake 7–9 times (Marschner et al. 1989). Overexpression of these two genes cannot be used to improve the tolerance of plants to Fe deficiency in alkaline soils, as metal uptake via IRT1 and Fe reduction by FRO2 are carefully regulated at the level of transcription and protein accumulation (Connolly et al. 2002, 2003). 35S-*FRO2* transgenic plants showed enhanced growth on low-Fe medium (pH 6.0) (Connolly et al. 2003), but FRO2 is not highly active under high pH conditions (Susin et al. 1996).

In the present report, we show that transgenic tobacco lines carrying the in-vitro-evolved *refre1* (372) gene, which is not regulated post-transcriptionally, are the first tobacco plants to display tolerance to low Fe availability in alkaline soils. In addition to Fe uptake by the roots, the unloading of Fe from the xylem sap is an important consideration in such lines. Fe is thought to be transported in the xylem as an Fe(III)-chelate complex, and therefore Fe(III) must be reduced before transport into the leaf cells. The ferric chelate reductase activity in the plant also appears to increase during seed development (Grusak 1995). Kosegarten et al. (1999) showed that in excised sunflower leaves, increased apoplastic pH decreases Fe^{3+} reduction. Taken together with these observations, the expression of the *refre1* variants under the control of the CaMV 35S promoter may enhance the transport of Fe in the vascular cylinder and in the aerial parts of the plant. We have also demonstrated the significance of nicotianamine in metal mobilization in plants (Takahashi et al. 2003). Introduction of the nicotianamine synthase gene (Higuchi et al. 1999) together with the *refre1* (372) gene has the potential to engineer plants that are more tolerant to low Fe conditions and that show increased productivity in alkaline soils; such plants may lead to increased crop production.

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